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FIBROBLAST GROWTH FACTOR AND DIABETIC RETINOPATHY

by

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A thesis submitted for the degree of
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University of London
Royal Postgraduate Medical School

For Jane

'Omnis cellula e cellula'

Where a cell arises there a cell
must have previously existed ...

Rudolf Virchow (1821 - 1902)
Cellular Pathology, Lect.II

ABSTRACT

Basic fibroblast growth factor (bFGF) is a potent angiogenic protein which is found in the retina and which may be involved in the development of proliferative diabetic retinopathy. We have developed sensitive immunoassays to measure bFGF and have established cultures of bovine retinal endothelial cells (BREC) to investigate the effects of growth factors on cell proliferation.

We have confirmed the potent growth stimulatory activity of bFGF, acidic fibroblast growth factor and retinal extracts on BREC but were unable to show a growth response of these cells to IGF-1 or an additive or synergistic effect between this growth factor and bFGF. Serum from patients with proliferative diabetic retinopathy did not show increased growth stimulatory activity for BREC compared to serum from controls.

Basic FGF was not detectable in human serum or vitreous samples from patients with proliferative diabetic retinopathy nor was there a difference in the levels of insulin-like growth factor 1 (IGF-1). However, bFGF was present in bovine retinal extracts, extracts of cultured BREC and in the extracellular matrix produced by these cells. In culture, the release of bFGF from BREC coincided with the release of lactate dehydrogenase indicating that cell membrane damage and lysis were responsible. IGF-1 was released by a different mechanism probably involving secretion.

Increasing the glucose concentration in the culture medium did not effect the proliferation of BREC or their response to added bFGF and there was no increase in the release of this growth factor or of IGF-1. Similarly, reducing the oxygen concentration in the culture medium did not alter the amount of bFGF subsequently released from BREC however it did promote increased cell proliferation.

These results indicate that bFGF could be released from damaged retinal cells and provides further evidence for the involvement of this growth factor in retinal neovascularization.

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STATEMENT OF COPYRIGHT

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PUBLICATIONS

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ABBREVIATIONS

ADP	Adenosine 5′-diphosphate
dATP	2′-Deoxyadenosine 5′-triphosphate
Bo	Label bound to antibody in the absence of cold antigen
BREC	Bovine retinal endothelial cells
BRP	Bovine retinal pericytes
BSA	Bovine serum albumin
CHAPS	3[(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonic acid
cpm	Counts per minute
CS	Calf serum
dCTP	2′Deoxycytidine 5′-triphosphate
C.V.	Coefficient of variation (mean/s.d x100)
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
cDNA	Complementary deoxyribonucleic acid
ECM	Extracellular matrix
ED_{50}	Dose producing 50% of maximum stimulation
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunoabsorbent assay
ESAF	Endothelial cell-stimulating angiogenesis factor
FCS	Foetal calf serum
aFGF	Acidic fibroblast growth factor
(r)bFGF	(Recombinant) basic fibroblast growth factor
GARS	Goat anti-rabbit serum
GTC	Guanidinium isothiocyanate
dGTP	2′-deoxyguanosine 5′-triphosphate
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid
HPLC	High performance liquid chromatography
HSPG	Heparan sulphate proteoglycan
IGF-1	Insulin-like growth factor 1
IRMA	Inter-retinal microvascular abnormality

I.U.	International Units
Kb	Kilobase
KDa	Kilodaltons
MEM	Minimal Essential Medium
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced form)
NAD(H)	Nicotinamide adenine dinucleotide (reduced form)
NIGS	Non-immune goat serum
NIMS	Non-immune mouse serum
NIRS	Non-immune rabbit serum
NSB	Non-specific binding
PBS	Phosphate buffered saline
PBSA	Dulbeccos 'A' phosphate buffered saline
PDGF	Platelet derived growth factor
PDS	Plasma-derived serum
PD-ECGF	Platelet derived-endothelial cell growth factor
PKC	Protein kinase C
PPS	Platelet poor serum
RIA	Radioimmunoassay
mRNA	Messenger ribonucleic acid
s.d.	Standard deviation
SDS	Sodium dodecyl (lauryl) sulphate
SEM	Standard error of the mean (s.d./$\sqrt{n}$)
TCA	Trichloroacetic acid
TGFß	Transforming growth factor beta
TGFα	Transforming growth factor alpha
TNFα	Tumour necrosis factor alpha
TPA	12-O-tetradecanoylphorbol 13-acetate
TPB	Tryptose phosphate broth
TRIS	2-Hydroxymethylpropane-1,3-diol
dTTP	2'-deoxythymidine 5'-triphosphate
VEGF	Vascular endothelial growth factor

Chapter 1. INTRODUCTION

1.1 The pathogenesis of diabetic retinopathy

Diabetic retinopathy is a micro-vascular complication of diabetes mellitus and is an important contributor to the morbidity associated with this condition. This has to be seen relative to the problems caused by other diabetic complications such as nephropathy and atherosclerosis. Diabetic retinopathy is a progressive disease with well defined stages and its rate of development varies between individuals. The prevalence of the later, more severe stages along with the success of the available treatments must be taken into account when assessing its impact on patient health.

1.1.1 Diabetes and its complications

Diabetes arises from the inability of the body to effectively remove glucose from the blood and extracellular fluid and metabolize it within the cells. The resulting hyperglycaemia has a major role in the development of the associated complications. There are two distinct types of diabetes. The first, type 1 diabetes, usually has an age of onset below 30 years and is due to the loss of pancreatic ß-cells by an auto immune mechanism. Since there is an absolute deficiency of insulin production, treatment is by insulin replacement therapy. Type II diabetes also involves changes to the pancreas and although insulin is produced, there is an altered insulin response to glucose (Temple et al., 1989). There is also resistance to insulin action at the tissues. Type II diabetes has an older age of onset and can be treated by diet, drugs or insulin therapy, it is also the more common form of diabetes with a prevalence, in Europe, estimated at 2% of the population (Gries, 1990).

Both forms can lead to the development of complications. These can be classified into four groups; neuropathy, affecting nerve function; macroangiopathy, mainly atherosclerosis; microangiopathy, sub-divided into

nephropathy affecting kidney function and retinopathy producing damage to the retinal vessels and; other pathologies associated with poor diabetic control, for example diabetic cataract formation, delayed wound healing and increased susceptibility to infection. Because of the high incidence of diabetes in the general population and the common development of complications, this illness produces a large amount of mortality and morbidity. Macroangiopathy and nephropathy are the major causes of mortality amongst diabetics but this does not diminish the problems caused by the other complications.

1.1.2 Diabetic retinopathy

The consequences of diabetic retinopathy depend upon its development in the individual. It may not progress beyond the retinal changes of background retinopathy which produce little or no effect on the patients visual acuity. Background retinopathy can develop into maculopathy, proliferative diabetic retinopathy or a combination of these. The former is more common in type II diabetes and is characterized by macular oedema. Proliferative diabetic retinopathy is characterized by the growth of new capillaries from existing vessels supplying the inner retina. There is associated fibrosis and vessels breaking through the inner limiting membrane are prone to haemorrhage with further fibrosis leading to traction retinal detachment. Its most aggressive form occurs in young type I diabetics. Both maculopathy and proliferative diabetic retinopathy are major causes of blindness. It is estimated that 30,000 to 40,000 people worldwide become blind each year from retinopathy (Merimee, 1990) and in the Western world it is the major cause of blindness amongst the working population (Grey et al., 1989). Therefore, although retinopathy does not produce the mortality associated with nephropathy and macroangiopathy, its importance is obvious with the potential for producing visual loss amongst young people.

Prevention has centred on improving the control of blood glucose, but this is not always easy and must probably be

instigated early in diabetes because many of the changes are not reversed by improving control at a later stage. Treatment of both maculopathy and proliferative diabetic retinopathy uses lasers to burn the layers of the inner retina and whilst this may be effective in arresting and reversing the progression of the disease, treatment may often have to be repeated many times and is not always successful. New treatments are therefore needed and an understanding of the biochemical mechanisms underlying the pathology is essential for their rational development.

1.1.3 The anatomical changes of diabetic retinopathy

The anatomical changes involved are now fairly well understood (Kohner & Dollery, 1975). One of the earliest findings is the selective loss of retinal capillary intramural pericytes. These cells lie within the basement membrane of the capillary endothelial cells. They are initially present in a one to one ratio with capillary endothelial cells and this ratio is the highest of any vascular bed in the body, perhaps indicating their importance in the retina. They may have a role in regulating capillary blood flow (Takahashi et al., 1989) and in maintaining the stability of the vessel wall (Cogan et al., 1961). They have also been shown to exert an inhibitory control on capillary endothelial cell proliferation in culture (Orlidge & D'Amore, 1987). Their selective loss in diabetic retinopathy may be responsible for some of the observed changes to the retinal circulation, to capillary permeability and may also enable the endothelial cell proliferation seen in microaneurysms and in the new capillary growth.

Another early change is the thickening of the capillary basement membrane (Ashton, 1974). Retinal endothelial cells secrete a basement membrane or extracellular matrix (ECM) from their basal surface. Retinal pericytes are situated within this matrix and they also secrete a basement membrane which surrounds the capillary. The matrix has a complex structure composed of collagen, laminin, enactin, fibronectin, thrombospondin and proteoglycans, particularly

heparan sulphate proteoglycan (Canfield et al., 1986) and its thickening in diabetes is a sign of the altered metabolism of the cells in the vessel wall. Basement membrane thickening probably predates pericyte loss and could prevent pericyte contact with endothelial cells thereby removing an inhibitory influence on endothelial cell proliferation. Microaneurysms are a distinctive feature of diabetic retinopathy and are a localised distension of the capillary wall which may be due to pericyte loss.

The structural changes described above are accompanied by an increase in vascular permeability. The blood supply to the inner retina is from branches of the central retinal artery which lies superficially in the nerve fibre layer (Fig 1.1). These arterioles supply a double layered capillary plexus.

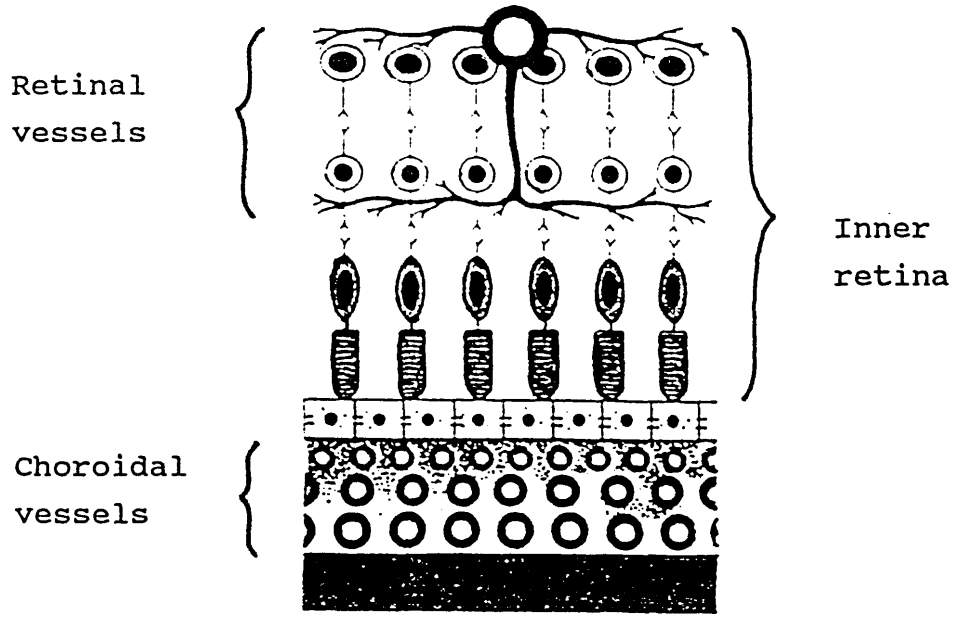


Fig. 1.1 The blood supply to the retina (Spalton et al., 1984)

The endothelial cells of these vessels form the inner blood retinal barrier with tight junctions between the cells called the zonae occludentes. The breakdown of the blood retinal barrier in diabetes was discussed by Cunha-Vaz et al., (1975). Capillary leakage is initially due to the abnormal

passage of small molecules through the endothelial cell itself (Kohner, 1990), although later there is thought to be a breakdown of the tight junctions. Leakage may also occur from microaneurysms. The leakage manifests itself as hard exudates of protein and lipid material on the retinal surface. In more advanced disease, fluid can accumulate producing retinal oedema and when this is around the fovea, it is responsible for diabetic maculopathy.

Retinal blood flow increases in early diabetic retinopathy (Kohner, 1976) and this may be due to autoregulation in response to decreased oxygen utilization caused by hyperglycaemia (Kohner & Porta, 1990). Increased blood flow could damage endothelial cells lining the vessels by shear stress (McMillan, 1978) and contribute to the progression of retinopathy. There is also the appearance of small retinal haemorrhages.

Another process that begins early in the development of diabetic retinopathy is progressive capillary closure. The endothelium normally presents a non-thrombogenic surface, however, changes in diabetes reduce its ability to carry out this function. Platelets are also altered and show increased coagulability (Porta et al., 1980). There is increased blood viscosity (McMillan, 1983) and decreased fibrinolysis (Brownlee & Cerami, 1981). These changes result in the formation of microthrombi in the capillaries and capillary closure which produces areas of retinal hypoxia. As these hypoxic areas increase there is the development of a recognized pre-proliferative stage characterized by venous beading, intra-retinal microvascular abnormalities (IRMA) and cotton wool spots. IRMA are possibly early new vessels (Mohan & Kohner 1986) whilst cotton wool spots are swellings of the nerve fibre layer due to interrupted axoplasmic transport secondary to hypoxia (Mcleod et al., 1979). Their appearance precedes the onset of proliferative diabetic retinopathy which is described in detail in Section 1.1.5.

1.1.4 Mechanisms of diabetic retinopathy development

Diabetic retinopathy is a progressive sequence of retinal

changes and the subsequent development of pre-proliferative and proliferative diabetic retinopathy is dependent upon the earlier events. Not all diabetic patients who progress to neovascularization develop all the retinal pathologies described above and some do not progress beyond background retinopathy.

The changes described reflect underlying biochemical and metabolic alterations in diabetes and probably the most important single factor is hyperglycaemia. Increased blood glucose results in high extracellular glucose levels and to uptake by those cells not dependent on insulin. There are several ways in which increased glucose is thought to lead to changes in the retina. Firstly there is increased activity of the sorbitol pathway in those cells containing the enzyme aldose reductase (Gabbay et al., 1966). Increased sorbitol flux may decrease levels of myo-inositol and reduce the activity of protein kinase C (PKC) and cell membrane sodium-potassium adenosine triphosphatase (Greene et al., 1987). Increased aldose reductase activity has been implicated in retinal pericyte loss (Buzney et al., 1977).

Non-enzymatic glycosylation of proteins is a major consequence of hyperglycaemia (Brownlee et al., 1984). If the turnover time of the protein is long, non-enzymatic glycosylation becomes irreversible and protein-protein cross-links can occur. This mechanism is thought to contribute to the increased basement membrane thickening seen in diabetes (Williamson & Kilo, 1979; Brownlee et al., 1988). Increased basement membrane component synthesis has been demonstrated in endothelial cells grown in high glucose (Cagliero et al., 1988) and this has now been shown to be due to upregulation of gene transcription which may be a consequence of the glycosylation of proteins involved in intracellular signalling or transcriptional control (Cagliero et al., 1991). The importance of ECM in cell differentiation, stabilization of phenotype and proliferation is well documented (Vracko, 1974; Wessels, 1977; Hay, 1981) and changes to the matrix may alter retinal capillary endothelial cell functioning and contribute to the development of diabetic retinopathy. Many other proteins are glycosylated in

diabetes, for example haemoglobin, tubulin and lipoproteins (Brownlee et al., 1984). This glycosylation may interfere with their correct functioning and also contribute to the changes seen in diabetic retinopathy.

The haemodynamic changes described in Section 1.1.3 are also important in the progression of diabetic retinopathy. In addition there are other metabolic and hormonal changes including increased circulating lipids and increased growth hormone, (Hansen, 1972, Hayford et al., 1980) which may effect basement membrane composition (Brosnan et al., 1971) and thrombus formation (Lufkin et al., 1979).

1.1.5 Mechanisms in the development of proliferative retinopathy - the involvement of growth factors

The retinal pathology described so far, pericyte loss, basement membrane thickening, capillary leakage, increased retinal blood flow, the changes in endothelial cell metabolism in response to hyperglycaemia and particularly the development of microthrombi leading to increased areas of hypoxic retina may all contribute to the progression to proliferative diabetic retinopathy. This is characterized by new blood vessels which originate mostly from the venous side of the circulation usually on or around the optic disk. They break through the inner limiting membrane and grow at the boundary of the retina and the posterior cortical vitreous gel. The latter may provide a scaffold on which the endothelial cells can proliferate. The new vessels are often leaky and develop tortuous paths. Their development is accompanied by the production of an epiretinal membrane of fibrous tissue which contains other retinal cells, retinal glia, fibroblasts and retinal pigment epithelium (Green et al., 1979). Bleeding from the new vessels and traction retinal detachment due to the accompanying fibrosis are the main causes of visual loss in proliferative diabetic retinopathy.

There are likely to be many factors contributing to the development of retinal neovascularization in diabetes. Normally endothelial cell turnover in the retinal vessels is

very slow (Engerman et al., 1967). Basement membrane changes, increased retinal blood flow and non-enzymatic glycosylation may all alter endothelial cell metabolism and increase their ability to respond to proliferative signals.

There is strong evidence for the involvement of growth factors, both circulating and locally produced, in proliferative diabetic retinopathy. A role for growth hormone was postulated after regression of retinopathy was observed in a 30 year old woman with poorly controlled diabetes following post-partum pituitary infarction (Poulsen, 1953), all known pituitary hormones were replaced except growth hormone. Subsequently, pituitary ablation was used as a treatment for diabetic retinopathy (Joplin et al., 1965; Bradley et al., 1965) and two controlled studies showed significant improvement, with amelioration of microaneurysms, haemorrhages and cotton wool spots (Lundbaek et al., 1969; Kohner et al., 1972) . There was marked regression of disk new vessels and pituitary ablation was particularly effective in the treatment of rapidly accelerating florid proliferative retinopathy (Kohner et al., 1976). The success of pituitary ablation as a treatment for proliferative retinopathy was correlated with the degree of growth hormone deficiency achieved (Wright et al., 1969).

Insulin-like growth factor 1 (IGF-1) is a basic peptide with a molecular weight of 7649 daltons which mediates the growth promoting activities of growth hormone (Schoenle et al., 1982). More than 90% of circulating IGF-1 is bound to a 150kDa binding protein, the production of which is positively regulated by growth hormone. Serum IGF-1 concentrations have been measured by RIA in diabetic patients and shown to be similar to those in normal subjects (Zapf et al., 1980; Lamberton et al., 1984; Hyer et al., 1988). It is interesting that in a 2 year prospective study there was a significant elevation of serum IGF-1 concentration at the time of the first appearance of new vessels. Levels returned to previous values after laser photocoagulation. (Hyer et al., 1989). IGF-1 may contribute to retinal angiogenesis following leakage from retinal capillaries or may be produced locally by the retinal tissues. It is now recognized to have

paracrine actions (Underwood et al., 1986).

Other growth factors that may be released from leaking vessels are those found in platelets; transforming growth factor ß (TGFß) and platelet-derived growth factor (PDGF) and also epidermal growth factor (EGF). Serum from diabetics and from those with diabetic retinopathy has been shown to be mitogenic for human umbilical endothelial cells in culture (Koh et al., 1985; Petty et al., 1988).

Locally released factors may play an important part in the stimulus to retinal endothelial cell proliferation. Several endothelial cell growth factors have been isolated from the retina or shown to be produced by retinal cells in vitro. They include acidic and basic fibroblast growth factor (aFGF and bFGF) (Baird et al., 1985a), TGFß (Antonelli-Orlidge et al., 1989), endothelial cell-stimulating angiogenic factor (ESAF) (Elstow et al., 1985) and factors released from retinal pigment epithelial cells (Wong et al., 1988). These are reviewed in more detail in Section 1.2.

1.1.6 Retinal hypoxia

The occlusion of retinal capillaries leading to the development of large ischaemic areas in the inner retina is a critical step in the development of proliferative diabetic retinopathy (Michaelson, 1948; Wise, 1956). The requirement for tissue oxygenation in the retina is greater than anywhere else in the body because of the very high metabolic rate of retinal tissues. Therefore, even a small decrease in the oxygen supply would result in retinal ischaemia (Sebag & McMeel, 1986). The importance of retinal hypoxia in the neovascular response is demonstrated by the fact that other conditions which predispose to proliferative retinopathy all produce ischaemic retinal tissue. These include central retinal vein occlusion (Magargal et al., 1981), sickle cell anaemia (Welch & Goldberg, 1966), retrolental fibroplasia (Ashton et al., 1954) and both Eales's disease and sarcoidosis (Wise, 1961). The release of a vasculogenic factor from ischaemic retina was first proposed by Michaelson in 1948. An angiogenic factor could be released

physiologically, for example in response to a decrease in pH due to increased lactic acid production or it could be released from retinal cells damaged due to the impairment of cytochrome oxidase activity and cell respiration (De Groot & Noll, 1987). The cells likely to be affected by hypoxia in diabetic retinopathy are those of the inner retina because cells in the outer nuclear layer; rods, cones and pigment epithelium receive oxygen from the choroidal circulation (Dollery et al., 1969; Linsenmeier, 1986) (Fig. 1.2).

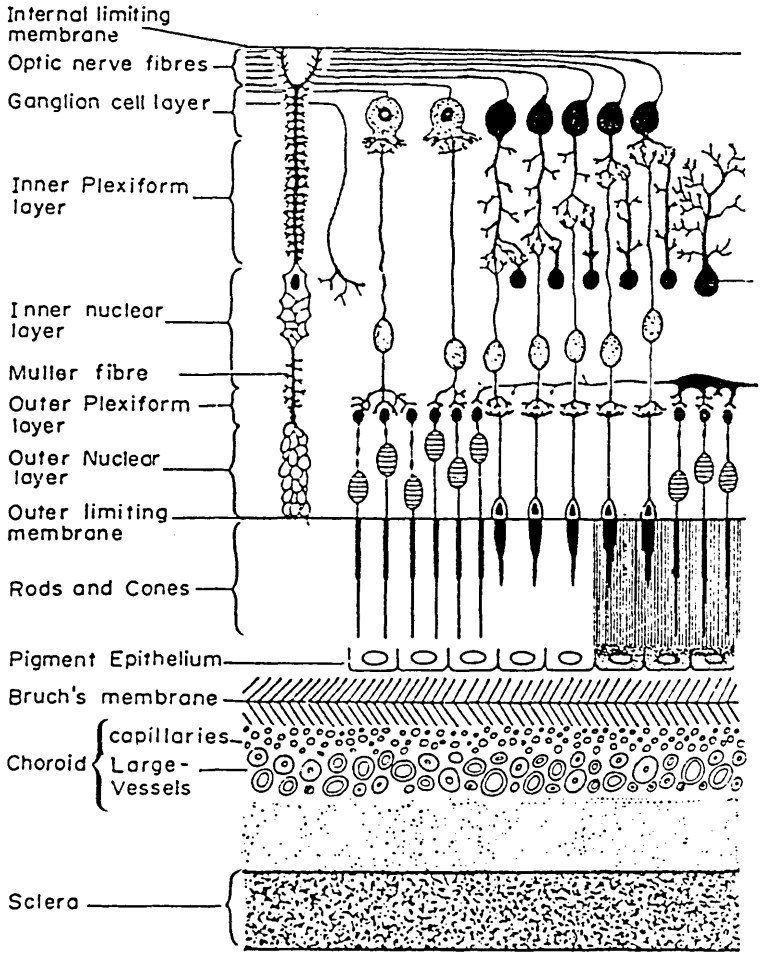


Fig 1.2 Cross-section of the retina (Trevor-Roper, 1974)

1.1.7 Angiogenesis

The term angiogenesis was first used in 1935 to describe the formation of new blood vessels in the placenta (Hertig, 1935). This process occurs physiologically in the developing embryo and in adult tissues during wound repair and in follicular, corpus luteal and placental development, in all these cases it is tightly regulated. Angiogenesis also occurs pathologically during the growth of solid tumours, in the development of proliferative retinopathy, in atherogenesis and in other disorders. In proliferative diabetic retinopathy the basic angiogenic process is the same as that seen physiologically except that it is not under the same degree of control.

Angiogenesis has been investigated using experimental models, the rabbit corneal micropocket technique (Gimbrone et al., 1974) and the chick chorioallantoic membrane (Ausprunk et al., 1975), which have allowed the observation of newly developing vessels. The development of biocompatible polymers for the sustained release of compounds allowed the testing of extracts and purified preparations for angiogenic activity (Langer & Folkman, 1976). Angiogenesis was shown to be a well defined series of events, (Ausprunk & Folkman, 1977; Folkman & Haudenschild, 1980; Folkman, 1985) see Fig. 1.3.

The first step in this process is probably an alteration in cell adhesiveness to the basement membrane and to adjacent cells (Forrester, 1987). This process may be influenced by changes in the composition of the ECM and by leakage of plasma components from vessels and the development of intravascular fibrin deposits (Brown et al., 1989). The next stage is the degradation of the surrounding ECM. New vessels mainly arise by sprouting from small venules (Ausprunk & Folkman, 1977) and must first breakthrough the surrounding basement membrane. Gross et al., (1983) proposed that this was due to the release of proteases. In particular, tissue plasminogen activator released by endothelial cells can convert plasminogen to plasmin, a serine protease which is able to degrade the ECM components fibronectin and laminin (Werb et al., 1980). Plasmin is also able to activate the

metalloproteases, type IV and interstitial collagenase (Salo et al., 1982; Werb et al., 1977). These would allow endothelial cells to penetrate the basement membrane and move into the intercellular stroma. Antibodies to tissue plasminogen activator, collagenase and type IV collagenase inhibited the bFGF stimulated invasion of bovine adrenal capillary endothelial cells into amnion basement membrane demonstrating a requirement for these proteases in basement membrane penetration (Rifkin et al., 1989).

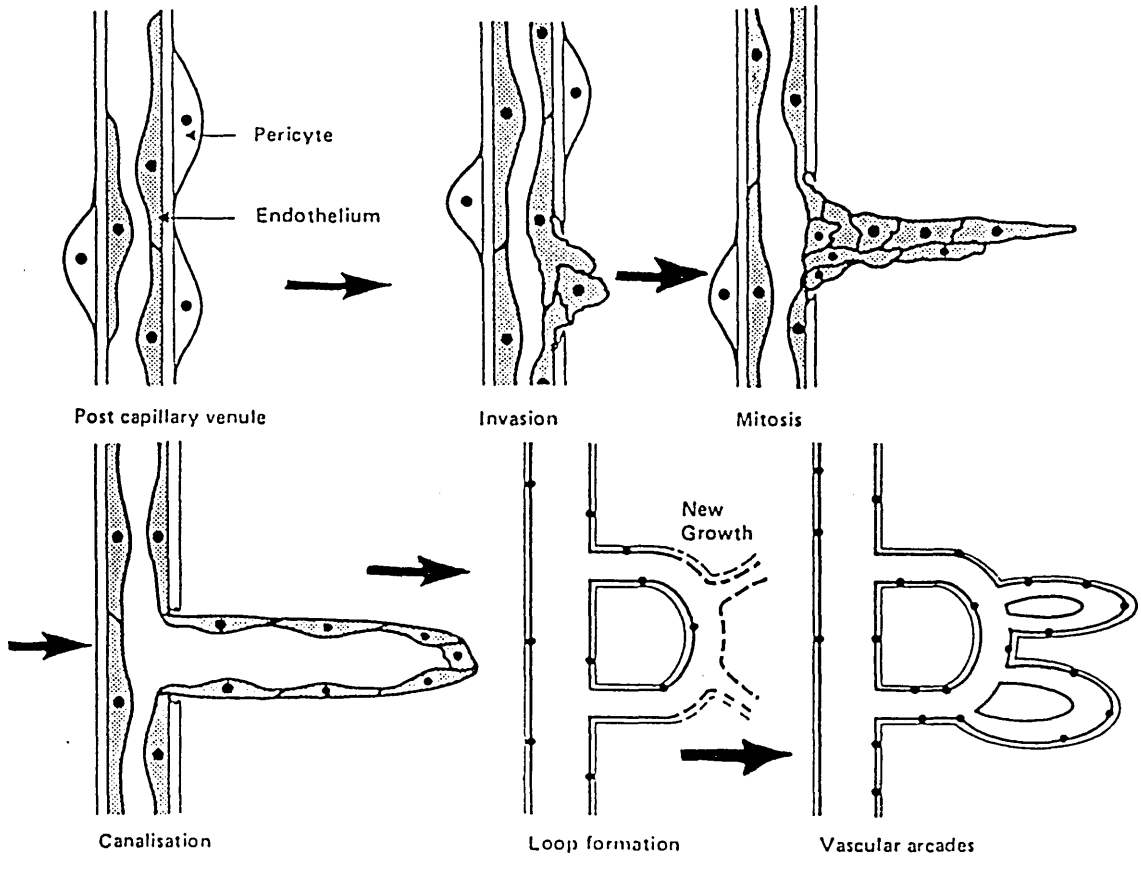


Fig. 1.3 The stages of angiogenesis (Forrester, 1987)

Migration of endothelial cells into the matrix is the next stage and chemotaxis of these cells towards various

stimuli has been investigated using assays of migration (Boyden, 1962; Ungari et al., 1985; McIntosh et al., 1988). Cells immediately behind these migrating cells proliferate to form a solid cord of cells. This process can occur in response to a non-mitogenic chemoattractant although at least in wound healing, migration and proliferation are thought to be due to two separate factors (Banda et al., 1982). A lumen is formed in this cord from a curve within the endothelial cells (Ausprunk & Folkman, 1977). The tip of the now hollow sprout of cells forms a loop which joins to a similar sprout. Secondary sprouts are then formed from these original loops producing a capillary network. An interesting feature of newly formed capillaries is the reappearance of pericytes (Herman & D'Amore, 1985).

1.2 Endothelial cell growth factors

The development of methods for endothelial cell culture (see Section 1.4) and models of angiogenesis (see Section 1.1.7) has led to the isolation of many substances capable of influencing endothelial cell growth. In vitro models have also allowed the testing of known hormones and cytokines some of which were found to effect endothelial cell proliferation. The sections below provide a brief overview of those factors which are well characterized or are of particular interest in diabetic retinopathy.

1.2.1 Purified growth factors and endothelial cell proliferation

Historically the first hormone with a proposed role in proliferative diabetic retinopathy was growth hormone. Growth hormone did not effect bovine retinal endothelial cell proliferation in one study (King et al., 1985). However it has since been reported that human growth hormone stimulated the proliferation of human retinal endothelial cells at concentrations seen in diabetic patients (Rymaszewski et al., 1991).

Another candidate as a endothelial cell mitogen in

proliferative diabetic retinopathy is insulin. Insulin treated diabetic patients are hyperinsulinaemic and combined insulin and pro-insulin fragment levels are high in type II diabetes (Temple et al., 1989). Insulin stimulates the proliferation of retinal and other capillary endothelial cells but not endothelial cells derived from large vessels (King et al., 1985; Bar et al., 1988). It has been suggested that insulin acts through IGF-1 receptors in promoting these mitogenic effects (King, 1985).

IGF-1 is a more potent stimulator of retinal capillary endothelial cell proliferation than insulin although it also has no effect on large vessel endothelial cells (King, 1985). The mitogenic effects of IGF-1 may be modulated by insulin-like growth factor binding proteins which are themselves produced by vascular endothelial cells (Bar et al., 1989a).

Three platelet derived factors have been shown to effect endothelial cell proliferation. PDGF stimulated ^{3}H-thymidine incorporation into microvessel endothelial cells and was also produced by endothelial cells (Bar et al., 1989b). Platelet-derived endothelial cell growth factor (PD-ECGF) was mitogenic for both large and small vessel endothelial cells (Miyazono & Takaku, 1990) whilst TGFß was an inhibitor of cultured endothelial cell proliferation (Muller et al., 1987). EGF stimulated the proliferation of bovine pulmonary and murine lung endothelial cells (Klagsbrun & D'Amore, 1991). Cytokines of the immune system have also been shown to affect endothelial cell growth. Interleukin-4 secreted by activated T-lymphocytes was a potent mitogen for capillary endothelium (Toi et al., 1991) and transforming growth factor α (TGFα), secreted by macrophages was also a mitogen (Klagsbrun & D'Amore, 1991) however tumour necrosis factor α (TNFα) which is also secreted by macrophages inhibited endothelial cell proliferation (Frater-Schroder et al., 1987). Some of these factors may be locally produced and have autocrine and paracrine actions.

Other locally produced factors which stimulate endothelial cell proliferation are acidic and basic FGF (see Section 1.3), vascular endothelial cell growth factor (VEGF)

isolated from bovine pituitary follicular stellate cells (Gospodarowicz et al., 1989) and ESAF, a low molecular weight angiogenic factor (Elstow et al., 1985) which is a specific mitogen for capillary endothelial cells and is found in both retina and vitreous (Taylor et al., 1989).

1.2.2 Circulating growth factors

Serum is a requirement for the growth of endothelial cells in culture. It is a complex mixture containing both growth and nutritional factors. Studies have shown that serum from diabetic patients can stimulate increased proliferation of cultured endothelial cells when compared to serum from non-diabetics (Koh et al., 1985; Petty et al., 1988). The factor responsible for this stimulation could be of importance in the development of proliferative diabetic retinopathy. A factor present in the serum of both type I and type II diabetic patients produced an increase in the proliferation of human umbilical vein endothelial cells derived from diabetic patients and non-diabetics (Koh et al., 1985). This finding was confirmed and a similar effect shown on human omental microvascular cells (Petty et al., 1988). The factor in the study of Petty et al., (1988) was heat stable and had a molecular weight of less than 15 kD.

1.2.3 Angiogenic factors

Many of the factors known to stimulate cultured endothelial cell proliferation are also chemotactic and angiogenic in experimental models. These factors are acidic and bFGF, VEGF, PD-ECGF and TGFß (reviewed in Klagsbrun & D'Amore, 1991). In addition however there are a number of substances which have no effect on endothelial cell proliferation but are angiogenic, for example, angiogenin (Strydom et al., 1985) and angiotropin (Hockel et al., 1988). Also TGFß and TNFα which inhibit endothelial cell proliferation in culture both show potent angiogenic activity (Muller et al., 1987; Frater-Schroder et al., 1987). These non-mitogenic and inhibitory substances may promote

angiogenesis in a variety of ways. Angiotropin for example, stimulates endothelial cell chemotaxis and TGFß attracts monocytes and macrophages (Klagsbrun & D'Amore, 1991) and could stimulate the release of mitogens from these and other cells. TGFß, TNFα and angiotropin also influence endothelial cell differentiation and promote the formation of tube like structures in collagen gels (Klagsbrun & D'Amore, 1991).

1.2.4 Retinal extracts and their characterization

The first demonstration of a vasoproliferative factor present in mammalian retina was the stimulation of foetal bovine aortic endothelial cells and corneal fibroblast proliferation and the induction of angiogenesis in the chick choriallantoic membrane by a bovine retinal extract (Glaser et al., 1980). The active component of this extract had a molecular weight greater than 12,000 daltons and was denatured by heating to 100^{0}C for 2 minutes. This factor was further characterised (D'Amore et al, 1981) and was called retina-derived growth factor (RDGF). It was shown by its retention on heparin-Sepharose and by its isoelectric point, (pI=5.0), to be similar if not identical to aFGF (D'Amore and Klagsbrun, 1984). Other groups also extracted and characterised endothelial cell growth factors from bovine and human retinae (Barritault et al., 1982; Courty et al., 1985; Lobb et al., 1986a; Lutty et al., 1986) and these were shown to be homologous to acidic and bFGF (Baird et al., 1985; Lobb et al., 1986b). The relative proportions of these two heparin binding growth factors in the retina were found to be different by these groups. Baird et al., (1985a) showed that greater than 85% of the bioactivity of the retinal extracts was due to bFGF whereas D'Amore & Klagsbrun, (1984) purified only aFGF. These differences were probably due to different extraction procedures and particularly the use of isoelectric focusing at pH 3.5-10 by D'Amore & Klagsbrun, (1984) as the first step in purifying the retinal extracts because this could have missed bFGF which has a pI of 9.6.

The compartmentalization of RDGF in bovine retina has been investigated (Plouet et al., 1986). They showed an even

distribution between inner layer neurones and photoreceptor cells but with a component tightly bound to the rod outer segments having a higher specific activity. They further characterised this factor as being homologous to aFGF on the basis of its retention on heparin-Sepharose and its protentiation by heparin (see Section 1.3.3) and they suggested a role for this factor in retinal signal transduction (Plouet et al., 1988).

The factors described so far were extracted from the neural retina. Vascular mitogens have also been shown to be released from retinal pigment epithelial cells (Wong et al., 1988) although the release of inhibitors of neovascularization from these cells has also been described (Glaser et al., 1987). This discrepancy was probably due to methodological differences in the assays systems used to detect these factors.

Other growth factors have also been described in retinal extracts. Chen & Chen (1987) have reported a specific vascular endothelial cell growth factor in foetal bovine retina which is distinct from the heparin binding growth factors and has a high molecular weight, whereas Kissun et al., (1982) described a low molecular weight growth factor in retinal extracts which can activate latent collagenase (Weiss et al., 1983).

1.3 Fibroblast growth factor

1.3.1 History

The last two decades have seen the isolation and purification of many growth factors with a wide range of target cells and biological activities. One of these was a factor present in vertebrate pituitary and brain. In the late 1960's and early 1970's a crude pituitary extract was found to be mitogenic for cultured fibroblasts and chondrocytes (Holley & Kiernan, 1968; Clark et al., 1972; Corvol et al., 1972; Armelin, 1973). In 1974 bFGF was isolated from crude pituitary extracts and characterised by its ability to stimulate the proliferation and alter the

phenotype of 3T3 fibroblasts (Gospodarowicz, 1974; Gospodarowicz & Moran, 1974). In 1975 aFGF was identified in brain extracts by its ability to stimulate myoblast proliferation (Gospodarowicz et al., 1975). Both of these factors have since been purified to homogeneity (Thomas et al., 1984; Gospodarowicz et al., 1985b) and have been isolated from and identified in many cells and tissues (see section 1.3.5).

Fibroblast growth factors were also isolated independently by a number of investigators and given many different names, heparin binding growth factor I and II, endothelial cell growth factor, eye derived growth factor I and II, RDGF and macrophage derived growth factor (Lobb & Fett, 1984; Maciag et al., 1978; Courty et al., 1985; Glaser et al., 1980; Leibovich & Ross, 1976). These factors have since been shown to be similar if not identical to acidic and bFGF (Baird et al., 1986; Thomas & Gimenez-Gallego, 1986) and at least 16 different synonyms are known for bFGF and 8 for aFGF (Gospodarowicz et al., 1987a) reflecting their wide tissue distribution and effects on many cell types.

1.3.2 Isolation and purification

The isolation and purification of bFGF was originally carried out from whole bovine pituitaries and made use of the cationic nature of the protein (pI=9.6) (Gospodarowicz, 1974, 1975). The steps were:-

1. Salt extraction at pH 4.5
2. Carboxymethyl Sephadex chromatography
3. Exclusion gel chromatography on Sephadex G75 followed by Biogel P-10.
4. Ion exchange chromatography on a Pharmacia mono-S cation exchange column.

This procedure resulted in a 200,000 fold purification and was also used to purify bFGF from bovine brain (Gospodarowicz et al., 1978a). Acidic FGF was purified from bovine brain (Thomas et al, 1984) using a slightly modified

method to that shown above. After Sephadex G75 chromatography, CM-Cellulose chromatography was used followed by isoelectric focusing. This step separated acidic from bFGF and the former was purified from ampholytes by reversed-phase HPLC using a Vydac C_4 column. Later modifications of the purification method for bFGF also used HPLC (Bohlen et al., 1984) and FPLC (Gospodarowicz et al., 1985b).

The purification method for both acidic and bFGF was greatly improved following the observation that these factors have a strong affinity for heparin (Shing et al., 1984). This led to a high yield, rapid, two-step purification method for these growth factors from bovine brain and pituitary (Gospodarowicz et al., 1984; Maciag et al., 1984; Lobb & Fett, 1984). Acidic and basic FGF were also purified by this method from hypothalamus (Klagsbrun & Shing 1985), cartilage (Sullivan & Klagsbrun, 1985) and retina (D'Amore & Klagsbrun, 1984; Baird et al., 1985a). Basic FGF alone was purified from corpus luteum (Gospodarowicz et al., 1985a), kidney (Baird et al., 1985b) and adrenal (Gospodarowicz et al., 1986a). The two steps involved in this method were cation exchange chromatography followed by heparin-Sepharose affinity chromatography. The fibroblast growth factors bound to the column were eluted with a stepwise or a linear gradient of NaCl with aFGF being eluted at a lower salt concentration than bFGF (Klagsbrun et al., 1987; Gospodarowicz, 1987b). The last step alone was responsible for a 2000-5000 fold purification of bFGF (Gospodarowicz et al., 1987a) which was considered pure as assayed by HPLC and by amino acid analysis(Baird et al., 1986).

1.3.3 Chemistry

The anionic nature of aFGF (pI=5.6) and the cationic nature of bFGF (pI=9.6) was the first known difference between these two growth factors. Gas-phase microsequencing resulted in the deduction of the primary structure of bovine pituitary bFGF (Esch et al., 1985a) which was a 146-amino acid protein. The primary structure of bovine brain aFGF soon followed (Esch et al., 1985b) and comprised 140 amino acids.

Comparison of the amino acid sequences showed that these two growth factors were very closely related with 55% structural homology and 64% of the remaining sequence possibly due to only one base substitution per amino acid. There was also sequence homology with interleukin-1ß and interleukin-1α (Thomas & Gimenez-Gallego, 1986) The calculated molecular weights of these factors were 16400 daltons for bFGF and 15800 daltons for aFGF and electrophoresis in polyacrylamide gels under denaturing conditions produced apparent molecular weights of 18 Kd and 16Kd respectively. However, both aFGF and bFGF from some isolations migrated as doublets (Thomas et al., 1984; Esch et al., 1985a). Examination of the amino acid sequences revealed the presence of amino-terminal truncated forms (Gospodarowicz et al., 1985a; Thomas et al., 1985)(see Fig. 1.4).

When bFGF extractions were carried out in the presence of protease inhibitors loss of the amino-terminal could be prevented (Mormede et al., 1985) and an NH_2-terminal extended form of bFGF was found (Ueno et al., 1986). Higher molecular weight forms of bFGF have also been detected by immunoblotting (Moscatelli et al., 1987; Presta et al., 1988; Doble et al., 1990; Grothe et al., 1990). Purification and sequencing of human basic and aFGF revealed a high degree of conservation between these and the bovine FGFs, with only two amino acid changes in the former and eleven in the latter (Sommer et al., 1987; Gimenez-Gallego et al., 1986).

Acidic and bFGF are globular proteins, their three dimensional structures have been crystallographically determined and contain 12 antiparallel ß strands (Zhu et al., 1991). They are labile molecules and lose biological activity below pH 3.5 and when heated to 70°C for 5 minutes (Gospodaowicz et al., 1978). Even at neutral pH and 37°C the measured in vitro half life of brain derived FGF was only 24 hours (Westall et al., 1983). This lability is thought to be due to the presence of only one disulphide bridge stabilizing the secondary structure (Yoshida et al., 1987). Basic FGF can form a further disulphide bond but this induces unstable, heterologous conformations. Serine substitutions at these cysteine residues stabilizes bFGF (Seno et al., 1988).

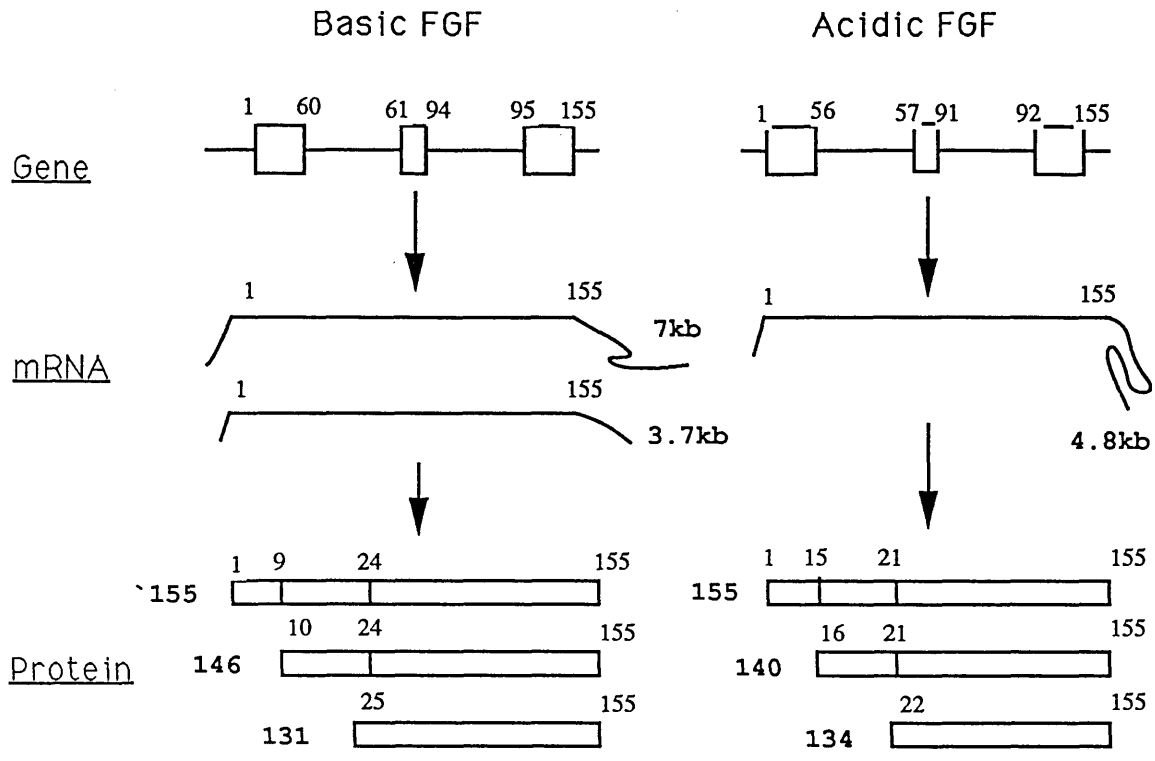


Fig. 1.4 Gene, messenger RNA (mRNA) and 1° structure of b and aFGF (Gospodarowicz et al., 1987b). The numbers refer the amino acid sequence and the mRNA transcript sizes are given in Kb

Heparin, a sulphated, heterogenous polysaccharide was found to protect a and bFGF from both acid and heat inactivation (Gospodarowicz & Cheng, 1986). Both a and bFGF contain two potential heparin binding domains (Esch et al., 1985a; 1985b) one close to the amino terminus and the other towards the carboxy terminus. By using synthetic peptides Baird et al., (1988) showed heparin binding by fragments derived from these or adjacent regions. It has been suggested that heparin could bind to these domains producing a cyclical molecule which would stabilize the structure of FGF (Gospodarowicz et al., 1987a). The relative affinity of these factors for heparin has been studied using heparin-Sepharose affinity columns and

eluting the growth factors with a gradient of increasing NaCl concentration. Acidic FGF was eluted at 0.9-1.1M NaCl, whilst bFGF has greater affinity eluting at 1.3-1.8M NaCl (Klagsbrun et al., 1987; Watanabe et al., 1990).

The hydrophobic nature of the fibroblast growth factors leads to their loss by absorption when in low concentration. A zwitterionic detergent 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS) was found to prevent this loss during storage and when used in experimental procedures (Matuo et al., 1988).

1.3.4 The gene and mRNA transcipts

The genes for both basic and aFGF have been cloned and mapped with restriction enzymes (Abraham et al., 1986a). Southern blotting showed only one bFGF and one aFGF gene. Their size is greater than 38Kbp and both code three exons separated by two large introns (see Fig. 1.4). Basic FGF is present on chromosome 4, specifically at 4q25 and aFGF on chromosome 5 (Abraham et al., 1987; Fukushima et al., 1990). If the genes for bFGF and aFGF are aligned so as to produce the greatest degree of homology, the location of the introns is identical and this suggests that they were derived from a single ancestral gene (Gospodarowicz et al., 1987b).

Complementary DNA (cDNA) clones for both basic and aFGF were isolated and their nucleotide sequences determined (Jaye et al., 1986; Abraham et al., 1986a; Kurokawa et al., 1987). They showed the translation initiation codons AUG and ATG respectively with the translated products comprising 155 amino acids in each case. This provided an explanation for the amino terminal extended forms of both acidic and basic FGF. The mature proteins are cleaved between amino acids 15 and 16 and 9 and 10 respectively with further proteolytic cleavage between positions 21 and 22 in aFGF and 24 and 25 in bFGF producing the truncated forms (Abraham et al., 1987) (see Fig.1.4). The cDNA cloning of a hepatoma derived growth factor and the transfection of COS cells with cDNA for bFGF in which the initiation codon had been altered demonstrated unconventional CUG initiation codons 5′ to the AUG codon

(Prats et al., 1989; Florkiewiecz & Sommer, 1989). This provided an explanation for the higher molecular weight forms of bFGF described in Section 1.3.3.

An interesting feature of the cDNA clones was the absence of a sequence coding for a classic signal peptide which would allow their secretion from cells. They possess specific cellular receptors (See section 1.3.7) and therefore have an extracellular function. This lack of a signal peptide in an extracellular protein is shared with interleukin 1 (March et al., 1985). An as yet undefined mechanism of release from cells is therefore likely (see Section 1.3.6).

Gene cloning has led to the discovery of five other members of the FGF family. Four of these are oncogene products; int-2 (Moore et al., 1986), hst/kFGF (Delli Bovi & Basilico, 1987; Yoshida et al., 1987), FGF-5 (Zhan et al., 1988) and FGF-6 (Marics et al., 1989). The fifth is a keratinocyte growth factor (Finch et al., 1989). All of these proteins show significant homologies to acidic and bFGF. The hst/kFGF and FGF-5 translated sequences contain signal peptides and the proteins may be involved in tumorogenicity and embryonic development (Rifkin & Moscatelli, 1989). However their role in non-transformed cells has yet to be defined.

Complementary DNA clones for both bFGF and aFGF were used as probes for mRNA transcripts and the initial studies showed undetectable levels in most tissues except for bovine hypothalamus and retina and human brain (Abraham et al., 1986a; Jaye et al., 1986; Alterio et al., 1988; Shimasaki et al., 1988). However, bFGF is widely distributed in many tissues (see Section 1.3.5) which led to the idea that bFGF mRNA is tightly regulated and may have a short half-life (Abraham et al., 1986b). Using reverse transcriptase-polymerase chain reaction amplification, mRNA for both basic and aFGF has been detected in rat ovary (Koos & Seidel, 1989; Koos & Olsen, 1989) indicating that FGF mRNA may be present in other tissues but at very low levels. FGF mRNA has also been measured in some neural tumour tissues (Murphy et.al., 1989a; Takahashi et al., 1990). In contrast to tissues, mRNA for bFGF has been detected in a variety of both normal cells

in culture and transformed cell lines (Murphy et al., 1988a; Sternfeld et al., 1988) including capillary endothelial cells (Schweigerer et al., 1987a; Bikfalvi et al., 1990). Transcript sizes ranged from 1.0 to 7.0Kb with the two major species being 3.7 and 7.0Kb. A possible explanation for multiple transcript sizes is transcriptional termination at several polyadenylation sites and this was supported by the finding of several poly(A) additional signals in cDNA clones for bFGF (Kurokawa et al., 1987). Incomplete processing or differential splicing of precursor mRNA could also be involved (Murphy et al., 1988b). Most of the work on bFGF mRNA regulation in cultured cells has been done with human astrocytoma cells. This has shown stimulation of bFGF mRNA by serum and an inverse relationship with cell density which is related to cell to cell contact and may explain the low mRNA levels seen in normal tissues (Murphy et al., 1988a). These cells however express high levels of bFGF mRNA. In a glioblastoma cell line expressing low levels, bFGF mRNA measurement was increased when mRNA transcripts were stabilized with the protein synthesis inhibitor cycloheximide, again suggesting that bFGF mRNA has a short half-life which may be responsible for the low levels seen in normal tissue (Murphy et al., 1990).

1.3.5 Localization and production

Basic FGF has been extracted and purified from a wide variety of normal and transformed tissues and from both normal and tumour cells in culture (reviewed in Gospodarowicz et al., 1987). By comparison, aFGF had, until recently only been detected in brain, retina and bone tissue and in osteosarcomas however the use of specific antibodies has demonstrated the presence of this growth factor in myocardial and skeletal muscle (Casscells et al., 1990; Morrow et al., 1990), Also, as described above, aFGF mRNA has been measured using very sensitive methods in rat ovary and has been detected in human umbilical vein endothelial cells. and abdominal aortic endothelial and smooth muscle cells by in situ hybridization (Mansson P-E et al., 1990) suggesting that

other non-neuronal tissues produce aFGF but that production is tightly regulated.

Basic FGF is synthesized by cultured endothelial cells from both large and small vessels (Moscatelli et al., 1986; Vlodavsky et al., 1987a; Baird & Ling, 1987) and this may account for bFGF extracted from some richly vascularized tissues although other non-transformed cells , for example, pituitary follicular cells, ovarian granulosa cells, adrenal cortex cells and myoblasts also contain bFGF. Two studies have examined the distribution of bFGF in normal human and tumour tissues using immunocytochemistry (Schulze-Osthoff et al., 1990; Cordon-Cardo et al., 1990). The first showed cytoplasmic staining of some endothelial cells present in lymph node, spleen and skin and staining of alveolar macrophages, keratinocytes, sweat and sebaceous gland cells and dendritic cells present in the placenta and thymus. The second study extended this range of cells to neurones and purkinje cells, bronchial, intestinal and endometrial epithelial cells and skeletal,myocardial and smooth muscle cells. These authors also showed homogenous staining in large and intermediate size blood vessels with heterogenous staining in capillaries. In contrast to the first study, bFGF was found ubiquitously in the basement membranes of blood vessels of all sizes and this difference could be due to the differing avidity and epitope specificities of the antibodies used.

There have been reports of bFGF in serum (Mormede et al., 1985; Baird et al., 1986) although the radioimmunoassay used to measure bFGF in these studies has since been invalidated (Gauthier et al., 1987). Recently bFGF has been detected in the serum of patients with multiple endocrine neoplasia type 1 by using a highly sensitive two-site immunoradiometric assay (Zimering et al., 1990).

Acidic and bFGF have been isolated from the bovine retina (see Section 1.2.4) and both cultured retinal pigment epithelial cells and retinal endothelial cells have been shown to synthesize bFGF (Schweigerer et al., 1987b; Bensaid et al., 1989a). The distribution of acidic and bFGF in rat and bovine retina has been investigated by cellular

fractionation, immunocytochemistry and in situ hybridization. Using the first of these methods aFGF was found to be tightly associated with the rod outer segment plasma membrane from bovine retinae (Plouet et al., 1988). In bovine retina, bFGF was localized around the capillaries of the inner nuclear layer (Hanneken et al., 1987) whereas aFGF was identified in the axons of the nerve fibre layer, in ganglion cells, Mueller cells, neurones of the inner and outer nuclear layer and in the inner and outer segments of the photoreceptors (Caruelle et al., 1989). In situ hybridization demonstrated aFGF mRNA in neuronal cells of the inner nuclear layer, ganglion cells, photoreceptors and pigment epithelium whereas bFGF was only found in the photoreceptors. Both acidic and bFGF have been shown to bind to Bruch's membrane and the inner limiting membrane in mouse embryonic eyes (Jeanny et al., 1987) and this evidence, along with the localization of aFGF to the inner and outer limiting membrane and bFGF to the basement membranes of retinal capillaries, suggests that these structures could serve as an extracellular storage site for fibroblast growth factors in the retina.

1.3.6 Interaction with the ECM

The lack of a sequence coding for a classic signal peptide in the mRNA transcripts of either acidic or bFGF which would allow their release from cells led to speculation as to their cellular localization and method of release. Several groups described the presence of bFGF in the ECM produced by aortic, adrenal cortex capillary and corneal endothelial cells (Vlodavsky et al., 1987b; Baird & Ling, 1987; Folkman et al., 1988) where it was bound to heparan sulphate proteoglycan (HSPG) (Saksela et al., 1988; Vigny et al., 1988). HSPG's consist of heparan sulphate chains covalently linked to a protein backbone. They are found in the plasma membrane and ECM of a wide variety of tissues and cultured cells (Nader et al., 1987) where they constitute greater than 90% of the glycosaminoglycan side chains (Kramer et al., 1982).

The matrix produced by vascular endothelial cells has

been shown to mimic the effects of bFGF on cells (Gospodarowicz et al., 1982). Also, large vessel endothelial and retinal capillary endothelial cells could be cultured in defined, serum-free medium on ECM produced by bovine corneal endothelial cells (Tauber et al., 1981; Gospodarowicz et al., 1986b). Although ECM contains several attachment and structural components, its effects on vascular endothelial cell proliferation and phenotype stabilization were not due to fibronectin, laminin or various collagen types (Gospodarowicz et al., 1983). In addition, ECM produced by PF-HR-9 cells, which do not synthesize bFGF, failed to support the growth of vascular endothelial cells (Rogelj et al., 1989). These results suggest that the effects of the ECM on proliferation and differentiation could be due to bound bFGF. Basic FGF bound to HSPG is protected from proteolytic inactivation (Saksela et al., 1988) but it can be released in an active form by treatment of matrices with heparinase or heparinitase enzymes (Bashkin et al., 1989; Presta et al., 1989). The presence of bFGF in the ECM led to the theory that this growth factor was translocated into the matrix along with HSPG (Baird & Ling, 1987).

Although bFGF and aFGF can be localized to the ECM of cells in culture and histologically to basement membranes from various tissues including those of the retina and the vasculature, the majority of bFGF in cultured vascular cells is present intracellularly where it can be extracted from cell lysates (Moscatelli et al., 1986; Schweigerer et al., 1987a; Vlodavsky et al., 1987b). The subcellular location of bFGF appears to be regulated by a signal sequence coded by a series of bases 5′ to the AUG initiation codon (see Section 1.3.4) so that proteins translated from the alternative CUG initiation codons are selectively localized to the nucleus (Bugler et al., 1991) and bFGF accumulation in the nucleus has been seen in cultured bovine aortic and adrenal capillary endothelial cells (Tessler & Neufeld, 1990).

Apart from the proposed translocation of FGF into the ECM, evidence has also been provided that bFGF can be released from endothelial cells by cell injury or lysis (Gajdusek & Carbon, 1989) or simply through mechanically

induced membrane disruptions (McNeil et al., 1989) after which it binds to the ECM. The matrix could act to buffer FGF levels and has been shown to prolong the activity of bFGF on cells in culture (Flaumenhaft et al., 1989; Presta et al., 1989). FGF present in the matrix could then be released from these stores by heparinase or heparinitase enzymes (Baird & Ling, 1987; Vlodavsky et al., 1987b) or by plasmin when it would remain bound to HSPG however, this did not effect its ability to interact with its receptor (Saksela et al., 1988).

1.3.7 Receptors

Specific receptors for bFGF were first identified and partially characterized on a baby hamster kidney cell line using ^{125}I-bFGF (Neufeld & Gospodarowicz, 1985). These cells had about 1.2 x 10^5 binding sites per cell, a receptor dissociation constant, K_D, of 270 pM and two receptor sizes with apparent molecular weights of 125 and 145 kD. This finding was extended to show that both basic and aFGF bind to the same receptor (Neufeld & Gospodarowicz, 1986). A single FGF receptor binding both acidic and bFGF was proposed by Olwin & Hauschka, (1986). Receptor populations for basic and aFGF have since been found on a variety of cultured cells (Friesel et al., 1986; Moscatelli, 1987; Kan et al., 1988; Olwin & Hauschka, 1989) with molecular weights ranging from 110-165 kD, K_D's from 8-270 pM and 700-120000 binding sites per cell. Some of these differences may reflect the experimental conditions used and higher values for the number of binding sites and low receptor affinities could be due to combined measurement of both high and low affinity binding sites (Moscatelli, 1987), the latter being cell associated heparin-like molecules. Differential glycosylation (Feige & Baird, 1988) could be responsible for some of the differences in receptor size. In addition, the affinity of the receptors for bFGF seems to be inversely related to the cellular content of this growth factor (Moscatelli, 1987). In contrast to cultured cells, high affinity receptors for FGF could not be detected on bovine tissue membrane preparations (Olwin & Haushcka, 1989) but were found on guinea pig brain membranes

(Ledoux et al., 1989) and rat hepatic plasma membranes (Gauthier et al., 1989).

The development of gene cloning techniqes and the use of expression vectors to transfect cell lines with cloned sequences has led to the isolation and characterization of several cDNA clones encoding fibroblast growth factor receptors (Johnson et al., 1990; Dionne et al., 1990; Houssaint et al., 1990; Miki et al., 1991; Hou et al ., 1991). These receptors are able to bind both acidic and bFGF. Two of these receptors are the products of genes designated flg and bek (Dionne et al., 1990) and two forms of flg differing in the number of amino terminal immunoglobulin-like domains are produced by alternative splicing of the gene product (Fujita et al., 1991). It is possible that in human liver cells up to 12 homologous receptors could be produced by differential splicing of the identified motifs for the ECM, transmembrane and intracellular domains (Hou et al., 1991). These receptor isoforms may be differentially expressed in different cell types, or at different stages in development and may have different binding affinities for acidic and bFGF or for FGF related oncogene products (see Section 1.3.4)

Glycosylation of the receptors has been shown to be necessary for their function (Feige & Baird, 1988) and in adrenal capillary cells, pretreatment with bFGF leads to a down regulation of receptor number (Tsuboi et al., 1990). Several groups have shown that when bFGF binds to microvascular endothelial cells it is internalized and is degraded by lysosomes (Moscatelli, 1988; Friesel & Macaig, 1988; Bikflavi et al., 1989). Protein phosphorylation is involved in the postreceptor signal transduction mechanism of FGF receptors and this appears to be mediated by PKC in the mitogenic and chemotactic response of bovine cerebral cortex capillary endothelial cells (Daviet et al., 1990). An involvement of PKC in bFGF receptor signal transduction in Swiss 3T3 cells along with calcium mobilization was described (Tsuda et al., 1985), although calcium increases were not found to be necessary for bFGF stimulation of mitogenesis in BALB/c3T3 cells (Tucker et al., 1989) and FGF could still

phosphorylate S6 kinase in 3T3 cells depleted of PKC by phorbol ester treatment (Pelech et al., 1986). A role for tyrosine kinases in protein phosphorylation has also been proposed (Coughlin et al., 1988) and some FGF receptors have been identified as products of the flg gene which encodes a protein tyrosine kinase (Dionne et al., 1990).

1.3.8 Biological and cellular effects

The effects of acidic and bFGF on cells and tissues both in vivo and in vitro are many and complex. They can be conveniently divided into their actions on cultured cells and their actions on biological systems in vivo.

1. Actions on cultured cells.

Both basic and aFGF are potent mitogens for normal diploid cells derived from the mesoderm and for several established cell lines, e.g. BALB/c3T3 fibroblasts, in culture. They also stabilize the phenotypic expression and induce differentiation in some of these cell types (reviewed in Gospodarowicz et al., 1987a). These effects are particularly apparent and have been studied extensively in corneal and vascular endothelial cells. Vascular endothelial cells from either large vessels or capillaries showed a proliferative response to bFGF concentrations as low as 0.1 pM with half maximal stimulation (ED_{50}) between 1.4 and 5.0 pM (Gospodarowicz et al., 1985b; Gospodarowicz et al., 1986c). Acidic FGF produces similar responses but it is between 30 and 100 times less potent depending on the cell type (Bohlen et al., 1985). In bovine retina-derived capillary endothelial cells the ED_{50} was 0.8 pM for bFGF and 158 pM for aFGF indicating an even greater difference (Gospodarowicz et al., 1986b). The reduced doubling time seen in these cultures is mainly due to a shortening of the G1 phase of the cell cycle (Gospodarowicz et al., 1981) and is linked to the rapid expression of the c-fos oncogene and a slower more sustained rise in the c-myc oncogene (Muller et al., 1984) both of which have been shown to be involved in cell proliferation

(Armelin et al., 1984). The effects of FGF on differentiation are most clearly seen with nerve cells where they promote neurite outgrowth in PC12 cell cultures (Togari et al., 1985) and can support the survival of neurones in primary culture (Walicke et al., 1986). In vascular endothelial cells they stabilize phenotypic expression possibly through control of the production of ECM components (Gospodarowicz & Tauber, 1980). They are also able to increase the lifespan of vascular endothelial cells in culture (Gospodarowicz et al., 1978b)

Both acidic and bFGF are chemoattractants for endothelial cells (Terranova et al., 1985; Conolly et al., 1987) including human capillary endothelial cells (Stokes et al., 1990) and release of bFGF was shown to be a requirement for the migration of bovine adrenal capillary endothelial cells (Sato & Rifkin, 1988). Basic FGF can also stimulate the production of both urokinase and tissue plasminogen activator by endothelial cells (Montesano et al., 1986; Sato& Rifkin, 1988) and plasminogen activation by these proteases enables bFGF to stimulate the invasion of three dimensional collagen matrices and human amniotic membranes (Montesano et al., 1986; Mignatti et al ., 1989).

Basic FGF may have a non-mitogenic role in both pituitary and ovarian function. It increases both the sensitivity of rat anterior pituitary cells to thyrotropin releasing factor and their release of thyrotropin and prolactin (Baird et al., 1985d). It also inhibits follicle-stimulating hormone mediated responses on rat granulosa cells (Gospodarowicz et al., 1987a).

2. Actions on biological systems in vivo

A role for basic FGF has been proposed in embryonic development, limb and lens regeneration, development of the nervous system and wound healing (reviewed in Gospodarowicz et al., 1987a and Schweigerer, 1988). In the retina, aFGF may be involved in signal transduction (Mascarelli et al., 1989) and bFGF may help in maintainance of the photoreceptors (Faktorovich et al., 1990).

The most widely studied effect of basic and aFGF in vivo is their ability to stimulate angiogenesis. A number of experimental models have been used to test the angiogenic effects of these growth factors including the chick chorioallantoic membrane (Thomas et al., 1985), the hamster cheek pouch (Gospodarowicz et al., 1986a), the rabbit corneal micropocket (Lobb et al., 1985) and the rat kidney capsule (Hayek et al., 1987) and results have shown that nanogram quantities of bFGF are able to elicit a response with aFGF slightly less potent. Angiogenesis is an important part of several physiological and pathological processes (see Section 1.1.7). The high concentrations of bFGF found in corpus luteum (Gospodarowicz et al., 1985a) suggest its involvement in the development of this tissue (Gospodarowicz et al., 1987a). Capillary growth at sites of tissue injury is also likely to involve bFGF (Schweigerer, 1988). Solid tumor growth is dependent on angiogenesis (Folkman & Klagsbrun, 1987) and the purification of bFGF from cultured tumour cells has led to the hypothesis that tumour progression is dependent on FGF (reviewed in Gospodarowicz et al., 1987a). However the finding that bFGF is not essential for the growth of highly vascularized solid tumours (Matzuzaki et al., 1989) suggests that tumour angiogenesis can be stimulated by other factors as well as by FGF and this heterogeneity is supported by the immunocytochemical localization of basic or aFGF to some tumour tissues but not to others (Schulze-Osthoff et al., 1990). There are many other diseases where angiogenesis is an important part of the pathology including rheumatoid arthritis, hemangioma, psoriasis, neovascular glaucoma and the proliferative retinopathies (Folkman & Klagsbrun, 1987) and FGF could be involved in these conditions.

1.3.9 Interaction with other factors

Both basic and aFGF interact with several factors involved in coagulation. As already discussed, heparin is capable of protecting both molecules from inactivation, it is also able to potentiate some of the actions of aFGF (Damon et al., 1989). Acidic but not bFGF is inactivated by thrombin

due to specific protein cleavage (Lobb, 1988) and bFGF binds irreversibly to α_2-macroglobulin in serum and plasma (Dennis et al., 1989). The α_2-macroglobulin-bFGF complex shows a reduced ability to bind to the FGF receptor and to stimulate plasminogen activator production. It is also rapidly removed from the circulation and this may be a mechanism for clearance of FGF released into the blood.

Basic FGF has been shown to interact with other cell growth factors. The individual actions of these factors on endothelial cell proliferation and the process of angiogenesis has been presented in Sections 1.2. However, it is likely that there is interplay between these factors in vivo in controlling endothelial cell proliferation and new capillary growth. Cell proliferation can be described by the cell cycle (see Fig. 1.5).

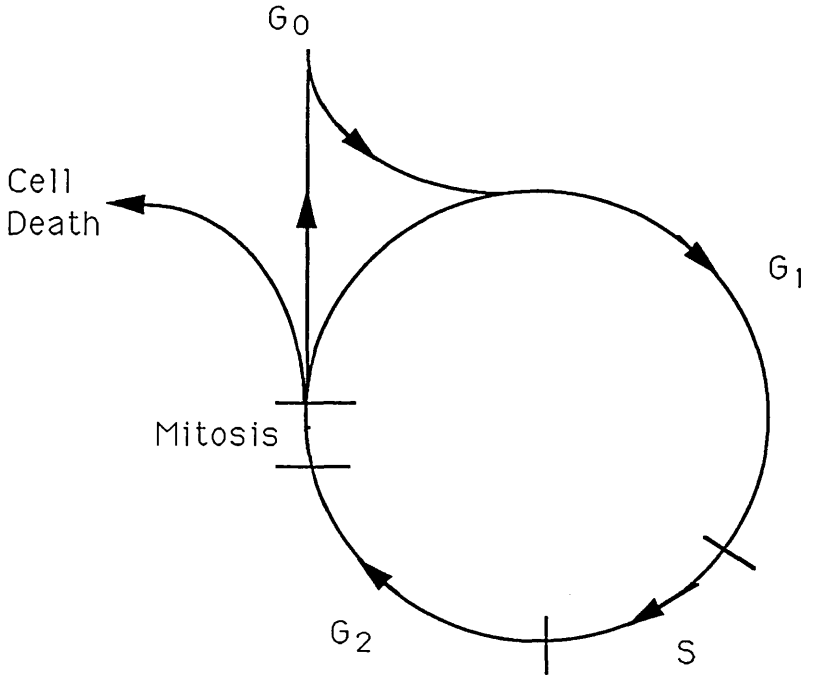


Fig. 1.5 The cell cycle

Non-proliferating cells are in a quiescent state (G_0) and control over whether a cell proceeds to DNA synthesis (S)

and, via an intermediate stage (G_2), to mitosis (M) can be exerted at two points (Pledger et al., 1977). The first point of control is between quiescence and the development of competence to enter the first stage of the growth cycle (G_1). The second point is during G_1 and permits progression to DNA synthesis. Growth factors have been grouped into either competence or progression factors depending upon the point at which they act. Basic FGF and PDGF have been described as competence factors and IGF-1, insulin and EGF as progression factors with one of each group necessary for cell proliferation (Van Wyk et al., 1981). However this work was carried out on BALB/c3T3 fibroblasts and the absolute requirement for both a competence and a progression factor for cell proliferation as well as their relative contribution to the mitogenic response may depend on the cell type (Ross & Ballard, 1988). In retinal capillary endothelial cells the mitogenic effect of bFGF or aFGF required the presence of serum factors and these were identified as high density lipoprotein and transferrin (Gospodarowicz et al., 1986b).

Another important concept in understanding the interaction of growth factors is that there is a balance between positive and negative forces on cell proliferation in a quiescent cell population (Pardee, 1987). This balance can be upset by either an increase in stimulatory influences or a decrease in inhibitory factors. TGFß is a potent inhibitor of endothelial cell proliferation (see Section 1.2.1) and its interaction with bFGF has been investigated. Basic FGF stimulated urokinase and tissue plasminogen activator production in adrenal capillary endothelial cells and this was inhibited by the concomitant addition of TGFß (Saksela et al., 1987). However, plasminogen activator inhibitor was also induced by both factors. Basic FGF altered the ratio of the mRNA levels of these protease activators and their inhibitor in favour of proteolysis whereas with TGFß the ratio would support prevention of proteolysis and explain the inhibition by TGFß of bFGF induced endothelial cell cords in fibrin gels (Pepper et al., 1990). In retinal capillary endothelial cell cultures TGFß inhibited bFGF-induced cell proliferation and its inhibitory potency was dependent on the

cell density (Bensaid et al., 1989b).

1.3.10 Measurement of FGF

The first measurements of bFGF were based upon its ability to stimulate the proliferation of BALB/c 3T3 fibroblasts (Gospodarowicz, 1975) and used the incorporation of methyl-^{3}H thymidine into newly synthesized DNA to quantify the response. The assay was used to monitor the purification of bFGF. A similar assay was used to monitor the purification of aFGF (Thomas et al., 1984). Bioassays involving other cell types and methods of measuring cell proliferation were used in subsequent purifications (Gospodarowicz et al., 1984; Lobb & Fett, 1984; Thomas et al., 1985; Baird et al., 1985a). A radioreceptor assay was developed for aFGF (Schreiber at al., 1985) and this group also developed an indirect enzyme-linked double antibody immunoassay for a FGF. A radioimmunoassay (RIA) and a solid phase enzyme immunoassay (EIA) were developed for aFGF which had sensitivities of 95 pM and 140 pM and cross-reactivities with bFGF of 0.79% and 0.84% respectively (Caruelle et al., 1988). The first RIA for bFGF was developed using antibodies raised against a synthetic decapeptide representing the amino terminal of FGF 1-146 (Baird et al., 1985c). The detection limit of this assay was 300 pM. Subsequently a solid phase RIA was developed using polyclonal antibodies against the intact bFGF molecule and this assay had a sensitivity of 400 pM (Joseph-Silverstein et al., 1988). RIA's and EIA,s for bFGF showed some cross-reactivity with aFGF and vice versa, probably due to the 55% structural homology between these molecules or to the contamination of the purified growth factor used for raising antibodies with the other growth factor species. An improvement in specificity was achieved with the development of a two-site enzyme linked immunoabsorbent assay (ELISA) for bFGF using monoclonal antibodies (Massoglia et al., 1987), however, the sensitivity was still no better than 350 pM. The ED_{50} for cell growth stimulation by bFGF can be as low as 0.8 pM (Gospodarowicz et al., 1986b) therefore the immunoassays described so far are all relatively insensitive. Recently two

site immunoradiometric assays for bFGF have been developed which make use of the affinity of this growth factor for heparin (Sato et al., 1989a; 1989b).These assays had sensitivities of 2 pM and 6 pM respectively and allowed the measurement of bFGF in medium conditioned by bovine corneal endothelial cells (Sato et al., 1989a) and in serum from patients with multiple endocrine neoplasia type 1 (Zimmering et al., 1990).

1.3.11 Involvement in proliferative diabetic retinopathy

Diabetic retinopathy is a progressive series of changes to the retina which are brought about by and reflect underlying changes in humoral and cellular biochemistry in diabetes (see Section 1.1). Changes in the production of fibroblast growth factors and in their interaction with other factors in the retina may occur throughout the development of diabetic retinopathy. However they are most likely to become involved during periods of cellular proliferation, perhaps transiently during the development of hypercellular microaneurysms and particularly during retinal angiogenesis. They may also be involved in the migration and proliferation of non-endothelial cells in vitreoretinal membranes.

There is a lot of circumstantial evidence that suggests that either basic or aFGF or both are involved in retinal new vessel growth seen in diabetes. Both these growth factors are present in the retina and can be extracted from retinal cell cultures in vitro. Both growth factors are angiogenic and can influence the three stages of angiogenesis in cultured cells; basement membrane degradation, endothelial cell migration and retinal endothelial cell proliferation. What evidence is there that these factors are involved in retinal neovascularization in vivo? Basic FGF injected into the vitreous cavities of cats produced a five fold increase in the incorporation of ^{3}H-thymidine into retinal cells when compared to bFGF that had been inactivated by boiling (De Juan et al., 1990). The labelling was predominantly in the venular endothelial cells (89%) with some labelling of capillary endothelial cells (10%). This would be consistent

with the observation that new blood vessel growth in the retina originates from the postcapillary venules (Michaelson, 1948). Another study however failed to show predominant endothelial cell staining in cat and rabbit retinae after intravitreal bFGF injection (Lewis et al., 1991) with glial cells more commonly labelled.

Studies using immunocytochemistry to localize both acidic and bFGF in epiretinal membranes removed from patients with proliferative retinopathy have produced differing results. Basic FGF was found in the basement membranes of established new vessels but not in those of developing vascular fronds (Hanneken et al., 1990). In other studies bFGF and aFGF were localized intracellularly with bFGF mainly in the nucleus and no aFGF detected in the ECM (Malecaze et al., 1991; Aotaki-Keen et al., 1991). The cell types most commonly stained were identified as macrophages and retinal pigment epithelial cells.

Basic fibroblast growth factor levels, measured by immunoassay, were elevated in vitreous removed from patients undergoing vitrectomy for proliferative retinopathy when compared to vitreous removed from patients with no neovascularization (Sivalingam et al., 1990). Increased concentrations of bFGF were particularly apparent in those patients with active proliferative retinopathy.

1.4 The use of cell culture models in diabetic retinopathy research

The use of cultured cells to study the cellular and biochemical changes involved in the development of diabetic retinopathy has been pursued for a number of reasons:-

(1) Although animal models of background retinopathy are available (Engerman & Kern, 1984; Sima et al., 1985) and new vessel growth has been described in galactosemic animals (Takahashi et al., 1991), there are no totally suitable models of diabetic retinal neovascularization.

(2) Cell culture experiments are relatively inexpensive and can be repeated many times adding statistical confidence to

the results.
(3) The experimental conditions can be carefully controlled.
(4) Cell culture models can be developed to screen for potential therapeutic agents prior to in vivo testing.

1.4.1 Cell culture methods

Since the early part of this century there have been attempts to culture endothelial cells from a variety of sources (reviewed in Jaffe, 1984). Outgrowths of cells from tissue explants survived for a few days only. The isolation of endothelial cells from trypsin treated human umbilical cord veins and rabbit aortas met with limited success because the endothelial cells did not replicate and the cultures rapidly became overgrown with contaminating smooth muscle cells and fibroblasts. Collagenase perfusion of veins from human umbilical cords provided the first reproducible method for the isolation of endothelial cells (Jaffe et al., 1973). Collagenase produced sufficient degradation of basement membrane to allow removal of the endothelial cells free of contaminating smooth muscle cells. These cells could be serially passaged. Bovine aortic endothelial cell cultures were subsequently established (Booyse et al., 1975).

Capillary endothelial cells proved more difficult to culture primarily because perfusion of the small vessels was not possible and collagenase digestion released both endothelial cells and pericytes with the latter cell type overgrowing in culture. The isolation and culture of capillary endothelial cells from the rat epididymal fat pad was reported in 1975 (Wagner & Matthews, 1975) and methods for the isolation of capillary endothelial cells from other tissues, including brain cortex, dermis and adrenal cortex followed (reviewed in Zetter, 1984). The basic method involved mechanical tissue disaggregation and enzymatic dissociation followed by sequential sieving on nylon membranes to remove larger vessels and single cells. Trapped microvessels could be further purified by velocity sedimentation on Percoll or albumin gradients. During the initial stages of culture, colonies of cells containing

pericytes could be removed by a process of weeding (Folkman et al., 1979) using a fine tipped pasteur pipette to scrape pericyte containing colonies from the culture dish.

Critical factors for the successful culture of endothelial cells were the growth medium used, the substrate upon which the cells were grown and the addition of growth supplements. The most favoured culture media have been Medium 199 (Jaffe et al., 1973), and DMEM (Folkman et al., 1979) supplemented with 10% or 20% serum. Large vessel endothelial cells will grow successfully on tissue culture plastic however capillary endothelial cells require the culture vessel to be precoated with a basement membrane component, usually gelatin, collagen or fibronectin (Zetter, 1981). Denuded ECM produced by corneal endothelial cells has also been used (Gospodarowicz et al., 1980). The use of growth supplements was found to improve the culture of endothelial cells and was found to be essential for the long term culture of capillary endothelial cells and for cell cloning. Supplements used have been those produced by feeder layers of mitomycin treated cells, tumour or other cell conditioned medium (Folkman et al., 1979) and brain extracts (Maciag et al., 1981) or purified fibroblast growth factors (Gospodarowicz et al., 1978b).

Metabolically active bovine retinal microvessels were first isolated in 1974 (Meezan et al., 1974) Subsequently retinal pericytes (Buzney et al., 1975) and retinal endothelial cells were cultured (Buzney and Massicotte, 1979; Frank et al., 1979). The methods used were essentially similar to those described above for capillary endothelial cells. Pericytes were selectively removed from the cultures by using the metabolic poison sodium ethyl mercurithiosalicylate (thiomersal) to remove these more rapidly mitosing cells. This method however resulted in toxic effects to the endothelial cells with consequent poor cell growth. Later methods for retinal endothelial cell culture involved the use of selective growth media and growth substrates. Bowman et al., 1982, used a fibronectin substrate and medium supplemented with plasma derived serum. The rationale behind the use of plasma derived serum was that it

prevented the release of platelet derived factors into the serum. Platelets have been shown to contain PDGF, a possible pericyte mitogen and TGFß, an endothelial cell growth inhibitor. Medium containing platelet poor plasma was also used in combination with a retinal extract to culture retinal capillary cells on a gelatin substrate (Gitlin & D'Amore, 1983). Retinal extracts have since been shown to contain fibroblast growth factor (see Section 1.2.4).

Two methods are now commonly in use for the culture of bovine retinal endothelial cells. One uses a gelatin or collagen substrate and medium supplemented with FGF, a brain extract containing FGF or cell conditioned medium. Alternatively a fibronectin substrate is used without the requirement for added growth factors. Refinements of the latter method for the selective culture of retinal endothelial cells have been described (Capetandes & Gerritsen, 1990). The culture of human retinal endothelial cells has proved to be even more difficult than those from other species however methods have recently been described (Grant & Guay, 1991).

Other retinal cells including retinal glia (Savage et al., 1988) and retinal pigment epithelium (Berman & Feeney, 1976) have been successfully cultured and are being used to model the cellular interactions occurring during the development of proliferative diabetic retinopathy.

1.4.2 Investigation of cellular interactions

Endothelial cells from both large and small vessels and from many vascular beds have been used to investigate factors which may be involved in the development of the vascular complications of diabetes. The results of these studies have yielded a large amount of data on the behaviour and metabolism of endothelial cells in culture. It is apparent from this work that whilst there are common features amongst endothelial cells for example, expression of factor VIII-related antigen, there are also significant differences. The differences between endothelial cells from large and small blood vesels has been reviewed (Zetter, 1981). There are also

differences in growth requirements between microvessel cells from different vascular beds (Marks et al., 1985). The species from which the endothelial cells are derived is also likely to be important in determining their properties.

The differences discussed above mean that the ideal cell type for the investigation of a particular pathology will be that derived from the tissue and species involved. For proliferative diabetic retinopathy this is human retinal endothelial cell and as described in the preceeding section, the culture of these cells is difficult and they have not been widely used. The information obtained from studies with other cells is however not invalid but must be treated with caution before using it in descriptions of disease development.

Endothelial cells and glucose

As discussed in Section 1.1.4 hyperglycaemia is likely to play a major role in the development of diabetic complications including diabetic retinopathy. The effect of glucose on endothelial cells cultured in medium containing different glucose concentrations has therefore been investigated. The glucose concentrations used for these studies were chosen to simulate those seen in the circulation of poorly controlled diabetic patients (20 mM) (Lorenzi et al., 1984) or a range of concentrations around this value (Porta et al., 1988). Mannitol, a non-metabolized hexose has been used to control for osmotic effects in some of these studies (Porta et al., 1988), galactose has been used to simulate polyol pathway metabolism and the L- isomer of glucose has been used to separate non-enzymatic glycosylation from metabolic effects (Cagliero et al., 1991). The majority of these studies have used human umbilical vein endothelial cells although bovine retinal capillary endothelial cells have also been used (Lee et al., 1989). Results have shown that high glucose produces delayed cell replication (Lorenzi et al., 1985; Porta et al., 1988) by prolonging cell cycle traversal (Lorenzi et al., 1987). Increased expression of basement membrane components has been observed (Cagliero et

al., 1988) and this has been linked to non-enzymatic glycosylation (Cagliero et al., 1991). Activation of a protein involved in cell signal transduction, PKC has also been shown (Lee et al., 1989), however high glucose had no effect on low density lipoprotein metabolism (Lorenzi et al., 1984) or on monolayer permeability of endothelial cells in vitro (King et al., 1987).

It is evident that high glucose has a wide range of effects on cell biochemistry and physiology and it is likely that reports of these disturbances will continue to grow as more studies are carried out. The direct relevance of these in vitro studies to the processes occurring in diabetic retinopathy must be considered with caution however as the length of exposure of these cell cultures to high glucose was not greater than two weeks whereas endothelial cells in the body are exposed to high circulating glucose levels for many years with a likelihood of even greater disturbances to their function.

Endothelial cells and hypoxia

The importance of hypoxia in mediating events subsequent to myocardial and cerebral ischaemia as well as its potential involvement in instigating new vessel growth in proliferative diabetic retinopathy (see Section 1.1.6). has led to studies on the influence of oxygen tension in the culture medium upon endothelial cell biochemistry and physiology. Several of these studies have used coronary or aortic endothelial cells, however umbilical vein endothelial cells and adrenal capillary endothelial cells have also been used. Many of these studies have been content to alter and monitor the oxygen levels in the gaseous phase during culture although a number have addressed the partial pressure of oxygen in the culture medium by blood gas analysis. When assessing the significance of experimental results it is necessary to take into account how the oxygen levels used relate to those observed in vivo. Oxygen tensions have been measured using micro electrodes placed above the retina and in the vitreous of diabetic patients undergoing vitrectomy (Maeda et al.,

1990). These have shown values of between 12.0 and 24.0 mmHg. However diffusion gradients across endothelial cells are higher than this and the oxygen tension in the retinal tissue will depend on its immediate locality, the proximity or otherwise of retinal arterioles and capillaries and the degree to which the latter are occluded.

From the experiments carried out it is evident that, with the qualifications discussed above, a decrease in oxygen tension can have marked effects on the functioning of endothelial cells in vitro. An increase in the sprouting of cells beneath confluent endothelial monolayers has been described (Smith, 1989) and stimulation of endothelial cell migration and proliferation by hypoxia has been shown (Meininger et al., 1988), an event that was ascribed to increased release of adenosine. Decreased ^{3}H-thymidine incorporation was however seen in both large and small vessel endothelial cells exposed to medium oxygen tensions of 21 mmHg and below for 48hrs, a treatment which was not lethally toxic to the cells (Shreeniwas et al., 1991). These low oxygen levels were also found to shift cell surface coagulant properties towards promotion of coagulation and to increase the permeability of cell monolayers to macromolecules (Ogawa et al., 1990). Hypoxia has also been shown to increase the release of endothelin, a potent vasoconstrictor from calf coronary artery endothelial cells (Hieda & Gomez-Sanchez, 1990) and to increase PDGF-B chain mRNA levels in human umbilical vein endothelial cells (Kourembanas et al., 1990).

1.4.3 Development of bioassays for growth factors using cultured cells

Bioassays for growth factors have measured the proliferative response of many cell types. Basic FGF bioactivity was measured using BALB/c 3T3 fibroblasts (Gospodarowicz, 1975). These cells are still used in many investigations because of the reproducibility of a transformed cell line and their ease of culture. Endothelial cells have also been used for example, human umbilical vein endothelial cells were used to assay for cell growth

stimulatory activity in serum from diabetic patients and from patients with diabetic retinopathy (Koh et al., 1985; Petty et al., 1988) and foetal bovine aortic endothelial cells were used to investigate the presence of endothelial cell growth factor activity in extracts and vitreous from normal and diabetic eyes (Lutty et al., 1986). The reference procedure for measurement of cell proliferation is cell counting with a Coulter electronic particle counter or a flourescence activated cell sorter although haemocytometer counting has also been used. This latter method was used to detect retinal vascular mitogens produced by retinal pigment epithelial cells (Wong et al., 1988). Counting methods are tedious and time consuming and other methods have been developed for measuring cell proliferation when large numbers of test samples are to be assayed. The most common method is ^{3}H-thymidine incorporation into newly synthesized DNA. The extent of cell replication can then be measured by counting labelled cells on an autoradiograph or by liquid scintillation counting of acid precipitable material. ^{3}H-thymidine incorporation was used in the initial characterization of retinal extracts using foetal bovine aortic endothelial cells (D'Amore et al., 1981). Another method for measuring DNA synthesis used staining with a flourescent dye (LaBarca & Paigen, 1980). The staining of cell protein has also been used as a measure of cell growth and proliferation. The original method used Coomassie brilliant blue G-250 dye staining of cells in 96-well tissue culture plates followed by spectrophotometry (Laughton, 1984) and this method has been refined to improve its sensitivity (McIntosh et al., 1988). Methylene blue dye has also been used in a similar assay for the assessment of growth factors (Oliver et al., 1989).

The detection and measurement of a cell growth inhibitor requires a bioassay system which is sensitive to the inhibitory effects of the factor. TGFß inhibits the proliferation of endothelial cells. However, bioassays used to quantitate this factor in human ocular tissues have used a mink lung epithelial cell line because of their ease of culture and sensitivity to TGFß's inhibitory effects (Connor

et al., 1989).

1.4.4 In vitro models of angiogenesis

A novel way of investigating the response of cultured cells to growth factors stemmed from the observation that muscle and adipose tissue explants embedded in collagen or fibrin gels produced outgrowths of cords of endothelial cells which formed capillary-like tubes (Montesano et al., 1985). It was also found that bovine retinal endothelial cells plated within a collagen matrix formed 3-dimensional networks of tube like structures (Schor & Schor, 1986). Endothelial cells plated on the surface of collagen or fibrin gels could be induced to invade the matrix by the addition of bFGF and phorbol esters (Montesano et al., 1986; Montesano et al., 1987) and formed tube-like networks of cells. This process was termed 'in vitro angiogenesis'. The inhibition of endothelial cell proliferation shown by TGFß in monolayer cultures was not evident when cells were plated in collagen gels, rather TGFß promoted cell to cell contact, the formation of junctional complexes and contraction of the gel (Merwin et al., 1990). Differences were also seen in the secretion and organization of the ECM when retinal endothelial cells were plated in collagen gels with a change in production of type IV collagen to types I and III and a decrease in the synthesis of thrombospondin (Canfield et al., 1986).

1.4.5 The limitations of the cell culture approach

The development of techniques for cell culture has enabled an enormous amount of information to be produced on the behaviour of cells grown under different conditions or in the presence of factors which are altered in diabetes. The same is true for the effect on retinal cells of factors thought to be involved in the development of diabetic retinopathy and the effect of growth factors on endothelial cell proliferation and the processes involved in angiogenesis. The results of these studies are of great

importance in understanding the biochemistry and physiology of these cell types and in providing clues to the mechanisms involved in the pathological process. One of the great strengths of cell culture is however also one of its weaknesses. Experiments involving one cell type and changing one parameter in the culture system whilst keeping others constant are essential if causes and effects are to be ascribed. This is an enormous oversimplification of the situation occurring in vivo. Cells are influenced by their environment and in culture this is artificial. Whereas care may be taken to provide matrix components and an optimum growth environment the surrounding milieu is likely to be very different to that in vivo and importantly, the influence of adjacent cells is lost. The very act of dissagregating tissue and stimulating cell growth in culture is likely to produce changes to the cell. For example, retinal endothelial cells have a very slow turnover in the vessel wall, however in culture they proliferate rapidly. Passaging cells is also likely to produce cellular alterations. A change in phenotype with repeated passage is recognized for several non-transformed cell lines with the loss of specific differentiated functions. Experiments are therefore often performed on cells of low passage number. The particular batch of cells used may effect their behaviour. The use of transformed cells whilst overcoming this batch to batch variation produces difficulty when extrapolating experimental results to the in vivo situation. These cells are likely to exhibit dedifferentiation with the loss of specific cell functions and the aquisition of some not associated with that cell type, for example, immortalization and a decrease in nutritional requirements.

The effects on cell behaviour of an alteration to one particular factor is difficult to relate to the in vivo situation where many such factors may be changing simultaneously. A tissue has many homeostatic mechanisms for the regulation of function and responses to a perturbance in function may occur at the level of the cell, at the level of the tissue and at the level of the organism where hormonal and other circulating factors are likely to be involved.

The problems of using data from cell culture experiments to describe in vivo pathology are not easy to reconcile. Probably the best that a cell culture model can say about a disease process is to describe what a particular cell type is capable of. This can lead to hypotheses which can be further investigated using pathological specimens and in vivo models. Cells in culture are also very valuable in detecting biological activity and the isolation and purification of many effector molecules involved in cell signalling has been made possible by their use. Another important use for cell culture models is to screen for potential therapeutic agents prior to in vivo testing.

One of the future directions for cell culture models in understanding pathological mechanisms is the use of co-culture techniques. These methods seek to investigate the cellular interactions occurring in vivo. This has already been done successfully for the interactions of capillary endothelial cells with pericytes and smooth muscle cells (Orlidge & D'Amore, 1987; Antonelli-Orlidge et al., 1989) and attempts have been made to construct artificial vessel walls using the appropriate cell types (Jones, 1979). Another way of increasing the relevance of cell culture systems by maintaining tissue architecture and cell to cell interactions is by the use of explants. An explant model of retinal cell proliferation has recently been produced (Forrester et al., 1990a).

1.5 Scope of the thesis

This thesis focuses on a crucial area in the development of proliferative diabetic retinopathy, the stimulus to neovascularization. The hypothesis is that during the development of diabetic retinopathy there is an alteration in the balance of growth influences on the retinal endothelial cells towards proliferation and that prior to the onset of new capillary growth there is the release of fibroblast growth factor from retinal cells. This potent angiogenic factor acts together with other growth stimulatory influences, both circulating and locally produced to promote

retinal endothelial cell proliferation and neovascularization.

The experimental work uses immunoassay, cell culture and Northern blotting to answer a number of specific questions:-

1. Is there growth stimulatory activity present in serum from diabetic patients with different grades of retinopathy when compared to diabetic patients without retinopathy and non-diabetic controls?
2. Is any serum stimulatory activity due to circulating bFGF?
3. Is bFGF or IGF-1 present in vitreous samples obtained at the time of vitrectomy from diabetic patients with proliferative diabetic retinopathy when compared to control subjects and are the levels of these factors altered?
4. Are bFGF mRNA transcripts detectable in cultured retinal endothelial cell extracts?
5. Is bFGF, a growth factor without a secretory signal peptide, released from retinal cells and if so under what circumstances?
6. How is the release of bFGF and the effect of this factor on the proliferation of retinal endothelial cells influenced by the glucose concentration of the cell culture medium?
7. Does oxygen tension in the medium influence retinal endothelial cell proliferation and bFGF release from these cells?

Chapter 2. MATERIALS AND METHODS

2.1 Developing a radioimmunoassay for basic fibroblast growth factor

Principle

An RIA for bFGF was set up using an antibody raised against a synthetic peptide containing the N-terminal 1-9 amino acids of $bFGF_{1-146}$. Subsequently a second RIA was developed using an antibody against a synthetic peptide, $bFGF_{1-24}$, which was the gift of Dr. A. Baird, Whittier Institute, La Jolla, U.S.A.. In both RIA's, a range of standards or unknown samples competes with ^{125}I-labelled bFGF for a limited amount of antibody. After incubation, bound and unbound label are separated by a double antibody precipitation technique and the bound fraction counted with a gamma counter. The amount of label bound is inversely proportional to the standard concentration and sample values are read from the standard curve.

2.1.1 RIA for bFGF using antibody raised against (Tyr^{10})FGF(1-10).

Assay buffer

Label, standard and antibodies were diluted in 10mM phosphate buffer pH 7.4 containing 150mM NaCl, 25mM EDTA, 200mM NaAcetate and 0.1% BSA (RIA grade, Sigma, Poole, U.K.), (FGF1-10 buffer).

Preparation of ^{125}I-(Tyr^{10})FGF(1-10)

A decapeptide having the N-terminal nine amino acids of $bFGF_{1-146}$ with the addition of tyrosine at position 10 ((Tyr^{10})FGF(1-10)) was synthesised by solid phase synthesis (Cambridge Research Biochemicals, Cambridge, U.K.). This peptide was iodinated by the chloramine-T method (Greenwood

et al., 1963). Chloramine-T oxidises $Na^{125}I$ to free ^{125}I allowing incorporation into tyrosine residues and the reaction is stopped by the addition of the reducing agent sodium metabisulphite. The following reagents were added sequentially, 10μl (Tyr^{10})FGF(1-10) (0.5 $mgml^{-1}$), 10μl 0.5M phosphate buffer (pH 7.4), 10μl $Na^{125}I$ (3700 $MBqml^{-1}$) and 25 μl chloramine-T (0.95 $mgml^{-1}$). The reaction was allowed to proceed for 15 seconds at room temperature and 50μl sodium metabisulphate ($2mgml^{-1}$) were added. Labelled peptide was separated from unlabelled peptide and free ^{125}I by reverse phase high pressure liquid chromatography (HPLC) with an isocratic gradient of acetonitrile containing 0.1% v/v triflouroacetic acid on a C18 column. Fractions containing labelled peptide were pooled and mixed 1:1 with freeze drying solution (2ml Trasylol/180mg BSA/8mg L-cysteine) and either stored frozen at -20°C or lyophilized in a freeze drier (Edwards High Vacuum, Crawley, U.K.).

Preparation of bFGF(1-10) antibody

a. Peptide conjugation

(Tyr^{10})FGF(1-10) was conjugated to bovine serum albumin (BSA) through tyrosine by bis-diazobenzidine conjugation. Fresh bis-diazobenzidine was prepared by stirring 10ml of an acidified solution of benzidine (2.5 $mgml^{-1}$) with 1ml $NaNO_2$ (11 $mgml^{-1}$) overnight on ice. BSA (6.7 mg) was dissolved in a bicarbonate solution (7 $mgml^{-1}$) containing the peptide neurotensin labelled with ^{125}Iodine (gift of Dr. M. Ghatei, Royal Postgraduate Medical School, London, U.K.) and mixed with (Tyr^{10})FGF(1-10) in a 1:4 ratio of protein to peptide. A 3-fold molar excess of bis-diazo benzidine was added and the pH adjusted to exactly 9.5 with 1N NaOH. The solution was stirred overnight on ice. To assess the success of the conjugation, unconjugated BSA/ ^{125}I-neurotensin was compared with the BSA/^{125}I-neurotensin/ (Tyr^{10})FGF(1-10) conjugate by mixing solutions containing the same number of counts per minute (cpm) with activated charcoal. The solutions were centrifuged and the cpm measured in the pellet and in the

supernatant. The solutions from successful conjugations were aliquoted into vials and freeze dried.

b. Production of antiserum

Immunization was originally carried out on 4 Dutch/Lop cross rabbits (Froxfield, Petersfield, U.K). A primary immunisation was prepared by mixing a 2:1 ratio of the peptide conjugate dissolved in tap water with complete Freunds adjuvant (85% hexadecane/15% alacia oil/1 drop killed mycobacteria). This was emulsified with a Ultra-Turrax Emulsifier (Janke & Kunkel, Staufen, Germany) and split into 4 aliquots. 50nM (starting peptide) of the conjugate was injected subcutaneously into each flank of each animal. Booster injections were prepared as for the primary immunisation except that incomplete adjuvant containing no mycobacteria was used and the quantity of peptide conjugate used for injection was halved. The first booster injection was carried out three months after the primary with subsequent booster injections every month. Sera were obtained by venepuncture of the marginal ear vein 7 and 10 days after each booster injection and stored at -20°C. A second immunisation program was subsequently carried out on 10 Dutch/Lop cross rabbits the procedure being the same as previously described except that the animals were split into 2 groups, one receiving the primary immunisation subcutaneously and the other by intradermal injections, also the first booster injections took place after 1 month.

Preparation of standards

A stock solution of (Tyr^{10})FGF(1-10) was prepared (2μM) and stored at -20°C. This was diluted in assay buffer to produce concentrations of 10-10,000 fmol/tube for use in antibody assessment and diluted 1:10 followed by double dilution (19.5-10,000 pmol) to produce a standard curve in the assay.

Antibody assessment

Antibodies were intially assessed by measuring total binding of neat antibody to $^{125}I(Tyr^{10})FGF(1-10)$. Labelled peptide was diluted in FGF1-10 buffer to give approximately 12,000 cpm. 100µl of this was mixed with 300µl of antibody and 100µl FGF(1-10)buffer in glass tubes, vortexed and incubated overnight at 4°C. Bound label was then precipitated with 2ml absolute ethanol and spun at 1400g for 60 minutes at 4°C. The supernatant was aspirated and the pellet counted in a NE 1600 gamma counter (Nuclear Enterprises, Edinburgh, U.K.). All antibody preparations showing binding to $^{125}I(Tyr^{10})FGF(1-10)$ were then titrated by testing serial dilutions in the same way. The antibody showing the highest titre was assessed for displacement of bound label with concentrations of $(Tyr^{10})FGF(1-10)$, (10-10,000 fmol/tube). This antibody was compared with an antibody to $(Tyr^{10})FGF(1-10)$ raised and donated by Dr. A. Baird.

Second antibody and non-immune serum optimisation

Ethanol precipitation of bound label was replaced by double antibody separation and this procedure was optimized by constructing a Latin square of non-immune serum and second antibody concentrations. A series of tubes containing label and buffer only (NSB) and containing antibody (1:1000), label and buffer (Bo) were incubated for 24 hrs. at 4°C and the bound fraction precipitated with a range of non-immune rabbit serum (NIRS) concentrations (1:10-1:40) coupled with a range of goat anti-rabbit serum (GARS) concentrations (1:10-1:40) (both sera from Biogenesis, Bournemouth, U.K.) for 96 hrs. at 4°C.

RIA procedure

The radioimmunoassay for bFGF was carried out as follows:- 100µl$(Tyr^{10})FGF(1-10)$ (19.5-10,000 pmol) or sample was added to 100µl antibody (1:10,000 final conc.) and 100µl label

(15,000 cpm) vortexed and incubated overnight at 4°C. 100μl NIRS (1:20) and 100μl GARS (1:50) were added and again incubated overnight. Tubes were then centrifuged at 1400g and the supernatant aspirated, the bound fraction was counted, a standard curve constructed of B/Bo versus log. bFGF concentration and the unknown sample concentrations read from this standard curve.

Assay optimisation

To optimise the assay the following procedures were carried out :-

a. The introduction of a 4 hour preincubation of standard and antibody prior to addition of label.
b. The effect of altering the label concentration (10,000-20,000 cpm/tube) on antibody titration curves.
c. The effect of altering assay incubation times.

An estimate of assay sensitivity was determined by setting up a series of Bo tubes in one assay.

Preparation and assay of samples

The optimised assay was used to investigate the concentration of bFGF in the following samples which were all stored at -20°C prior to use:-

a. Serum and heparinised plasma obtained by venepuncture from diabetic and non-diabetic individuals.
b. Vitreous samples obtained from diabetic patients with proliferative retinopathy and non-diabetic patients at the time of vitrectomy (Moorfields Eye Hospital, London, U.K.).
c. Rat sera, vitreous and retinal extracts obtained from Sprague-Dawley rats by dissection. Vitreous and retina were weighed and homogenised in 0.15M $(NH_4)_2SO_4$ prior to assay.

2.1.2 Measurement of aminopeptidase activity and its inhibition

The presence of an aminopeptidase in samples of human sera

and plasma and rat sera, vitreous and retinal extracts was determined by its ability to degrade and prevent $^{125}I(Tyr^{10})FGF(1-10)$ binding to the antibody.

Aminopeptidase in rat tissue extracts

Aminopeptidase activity in rat sera, retinal and vitreous extracts was assessed by incubation with $^{125}I(Tyr^{10})FGF(1-10)$, sampling at 0 and 2 hrs. and measuring specific binding to antibody after immunoprecipitation.

Inhibition of aminopeptidase activity in human plasma

Samples of human plasma were tested for inhibition of aminopeptidase activity by using the assay procedure in the presence of one of the following :- ethylenediaminetetra-acetate (EDTA) (4.1mM), trasylol (400 $I.U.ml^{-1}$), lima bean trypsin inhibitor (10 μgml^{-1}), bacitracin (0.8 $mgml^{-1}$) or phenylmethyl sulphonyl flouride (80 μgml^{-1}). To establish whether antibody binding was affected, a standard curve was prepared in the presence of a cocktail of these inhibitors. To further investigate the inhibition of enzyme activity by bacitracin and EDTA, human EDTA plasma samples containing a range of bacitracin concentrations (0.8-8 $mgml^{-1}$) were mixed with $^{125}I(Tyr^{10})FGF(1-10)$, diluted in assay buffer and incubated at room temperature. At times 0,1,2,4,6 and 28 hrs. 100μl aliquots were removed and label separated from enzymatic activity, M.W. 240,000 daltons (Gauthier et al. 1987) on Sephadex G25 (Pharmacia, Milton Keynes, U.K.) columns (6.5 x 0.6 cm). The labelled fractions were pooled, immunoprecipitated and the bound fractions counted.

2.1.3 RIA for bFGF using antibody raised against bFGF(1-24)

Assay buffer

All assay reagents except where indicated were diluted in 50mM phosphate buffer containing 175mM NaCl and 75mM EDTA to pH 7.4. This stock buffer was stored at 4°C and had 0.2% w/v

porcine gelatin, 200 bloom (Sigma) added which was dissolved by warming on a hot plate. The buffer was cooled before use (FGF1-24 buffer).

Preparation of standards

Bovine recombinant bFGF (rbFGF) (Amersham International, Aylesbury, U.K.) was used to prepare standards. The freeze dried preparation was diluted in sterile distilled water to 61 nmolml^{-1} and then further diluted in gelatin-free FGF1-24 buffer to 2 nmolml^{-1}. 5μl aliquots containing 10 pmol were stored frozen at -20°C. For use, one aliquot was diluted to 5 pmol/ml and then double diluted so that 100μl added to each assay tube produced a standard range from 0.975-500 fmol/tube. Standard diluent was FGF1-24 buffer, however other diluents were used depending on the samples being assayed. For bovine retinal endothelial conditioned medium, serum free Dulbecco's modified Eagle's medium was used , for dialysed heparin-Sepharose column fractions, eluting buffer dialysed in the same way was used and for vitreous samples, bovine vitreous was used. Using the appropriate diluent allows for the effect of sample composition on specific and non-specific binding to be eliminated.

Preparation of ^{125}iodine labelled rbFGF

Recombinant bFGF was originally iodinated by the lactoperoxidase method. This was then compared to the chloramine-T method and subsequently this latter method was used. Separation of bound from free ^{125}iodine was carried out by affinity chromatography although reverse phase HPLC was tried in an attempt to improve the specific activity of the label.

a. Lactoperoxidase method

Recombinant bFGF (0.3 nM) was diluted with 10μl ·0.1M NaAcetate buffer pH 5.0. 10μl $Na^{125}I$ (3700 MBqml^{-1}) was added followed by 10μl lactoperoxidase (0.4 units, pyrogallol

substrate, Sigma.) and 10μl hydrogen peroxide (0.03%). The reagents were mixed and left for 15 minutes during which time the enzyme oxidises $^{125}I^-$ to $^{125}I^+$ which is then incorporated into tyrosyl or histidyl residues. The reaction was stopped by the addition of 180μl 10mM Tris/HCl pH 7.4 containing 0.2% gelatin followed by immediate chromatographic separation.

Labelled rbFGF was separated from free ^{125}Iodine by heparin-Sepharose affinity chromatography as described in section 2.2.2. Purified label was diluted 1:1 with 50 mM phosphate buffer, pH 7.2 containing 0.2% gelatin and stored at 4°C. It was repurified before use in each assay using the same separation method.

b. Chloramine-T method

Recombinant bFGF (0.12 nM) was diluted in 10 μl 0.25M NaPhosphate buffer (pH 7.4). 10μl $Na^{125}I$ (3700 $MBqml^{-1}$) was added followed by 10μl chloramine-T (1 $mgml^{-1}$) as the oxidising agent. After 15 seconds the reaction was stopped by the addition of 100μl L-cysteine (0.54 $mgml^{-1}$) followed by 10μl potassium iodide (10 $mgml^{-1}$) and 250μl 0.05M NaPhosphate buffer (pH 7.4) containing 0.02% gelatin. Separation, storage and repurification of ^{125}rbFGF were as described above.

Comparison with commercially available ^{125}rbFGF

Recombinant bFGF labelled by the chloramine-T method was tested against a commercial product (Amersham International, Aylesbury, U.K.) by comparing standard curves set up with each label.

c. HPLC method

Reverse phase HPLC as a method of label purification was assessed by running ^{125}rbFGF on a C18 column with an isocratic gradient of acetonitrile containing 0.1% triflouroacetic acid.

Antibody assessment

Antibody RB773B15 raised in rabbits against bFGF(1-24) was a gift from Dr. A. Baird. On reciept, the antibody was diluted 1:20 with a 1:10 solution of non-immune rabbit serum, sterilised through an 0.2μ filter (ICN/Flow, Rickmansworth, U.K.) and stored at -20°C. A further dilution of 1:10 in gelatin free FGF1-24 buffer was used to provide a working stock solution and was stored in 100μl aliquots at -20°C.

Antibody titre was assessed by measuring binding of antibody to ^{125}rbFGF. A series of antibody dilutions (1:5000-1:32000) were prepared and 100μl of each incubated with 100μl ^{125}rbFGF diluted to give 15,000 cpm and 100μl FGF1-24 buffer overnight at 4°C. Bound label was precipitated by adding 100μl each of NIRS (1:20) and GARS (1:50), incubating for 4 hours at 4°C and spinning at 1400g followed by aspiration of the unbound label.

Second antibody and non-immune serum optimisation.

A Latin square was constructed to optimize the NIRS and GARS concentrations. A series of Bo tubes i.e. no unlabelled bFGF, was set up using 100μl 1-24 antibody, 100μl ^{125}rbFGF and 100μl FGF1-24 buffer along with NSB tubes containing label and buffer only. The tubes were incubated overnight at 4°C and the bound fraction precipitated with a range of NIRS concentrations (1:10-1:60) coupled with a range of GARS concentrations (1:10-1:60) and counted.

RIA procedure

100μl rbFGF or sample was added to 100μl antibody (1:20,000) and 200μl of label (12,000 cpm) diluted in NIRS (1:40), vortexed and incubated overnight at 4°C. 100μl GARS (1:50) was added and tubes incubated for 4 hours at 4°C. Tubes were then centrifuged at 1100g for 20 minutes and the supernatant aspirated. The bound fraction was counted and a standard curve constructed of B/Bo against log. bFGF concentration.

Assay optimization

The assay was optimized by keeping all other parameters constant and altering :-

1. Label concentration between 6000 and 12000 cpm/tube added
2. Buffer pH between 6.8 and 7.6
3. Incubation time after antibody addition (1.5-22hrs.) and after precipitating antibody addition (2-24hrs.)
4. The addition of a pre-incubation stage by adding sample or standard to the antibody before the addition of the label

Assay sensitivity was estimated by setting up a series of Bo tubes.

Cross-reactivity of other compounds in the assay

The cross-reactivity of the following compounds in the assay was assessed:-

1. Acidic FGF	0.04 -323nM
2. IGF-1	0.134-134nM
3. Adrenalcorticotrophic hormone	0.02-20 $ngml^{-1}$
4. Corticotrophin releasing factor	1-1000 $ngml^{-1}$
5. Human growth hormone	1-1000 $mIUl^{-1}$

2.2 Heparin-Sepharose chromatography

Principle

Affinity chromatography makes use of the high affinity of a compound for a binding agent coupled to a solid support in order to separate the compound from a mixture. The compound can also be characterised by its elution profile after elution with, for example, a chaotropic agent. In this case the high affinity of bFGF for heparin was used to separate ^{125}rbFGF from free ^{125}I after iodination and also to obtain

elution profiles of a retinal extract and medium conditioned by retinal endothelial cells. Agarose beads covalently bound to heparin were packed into a column, the mixture containing bFGF was run onto the column in a low salt buffer and then eluted from the column with either a stepped increase or an increasing concentration gradient of NaCl.

2.2.1 Heparin-Sepharose preparation

The method was the same for each application. Each gram of heparin-Sepharose (Pharmacia) required was swollen in 5ml 0.05M Tris/HCl pH 7.4 and then washed with 200ml of the same buffer on a sintered glass filter. It was stored at 4°C prior to use.

2.2.2 Purification of bFGF label

A glass pasteur pipette was prepared with a plug of glass wool in the bottom and was then half filled with swollen heparin-Sepharose. The beads were then washed with 12ml 0.05M Tris/HCl pH 7.4 containing 2.0M NaCl followed by 12ml 0.05M Tris/HCl pH 7.4 containing 0.15M NaCl. The mixture containing iodinated rbFGF (see Section 2.1.3) was applied to the column. The column was then washed with 12ml 0.05M Tris/HCl pH 7.4 containing 0.15M NaCl to remove unincorporated ^{125}I after which iodinated bFGF was eluted from the column with 0.05M Tris/HCl pH 7.4 containing 2.0M NaCl and was collected in 1ml fractions. The activity of the fractions was measured with a gamma counter and those containing the peak activity were pooled and a 1:1 ratio of 0.05M phosphate buffer pH 7.2 containing 0.02% gelatin was added. Repurification was carried out by repeating the above method before the label was used in the radioimmunoassay.

2.2.3 Heparin-Sepharose profile of rbFGF

A chromatographic tube, 150 x 9 mm (Column K9, Pharmacia) was packed with swollen heparin-Sepharose beads which were then equilibrated with 5 column volumes (40ml) 0.05M Tris/HCl

pH 7.4 containing 0.1M NaCl. The equilibration solution was allowed to run through the column by gravity until the bed surface just became dry. 1ml of 0.05M Tris/HCl pH 7.4 containing 0.1M NaCl and 305 pM bFGF was applied to the column and allowed to run on. A linear concentration gradient of NaCl (0.1-3M) in 0.05M Tris/HCl was produced and run through the column at 40mlhr^{-1} using a three-channel peristaltic pump (P-3, Pharmacia). 2ml fractions were collected using a Frac-100 fraction collecter (Pharmacia) and the fractions were stored frozen at -20°C prior to assay for bFGF. 10μl aliquots of each fraction were assayed which dilutes the NaCl sufficiently to prevent it from interfering in the assay.

2.2.4 Affinity chromatography of retinal extracts

Preparation of retinal extracts

Forty bovine neural retinae were removed from fresh bovine eyes as described in Section 2.5.1. They were placed in Dulbecco's A Phosphate Buffered Saline (PBSA) (Oxoid, Basingstoke, U.K.) containing 0.2mgml^{-1} glucose (1ml per retina) and left to soak for 2hrs at room temperature. The solution was then spun at 670g for 10mins and the supernatant removed and respun at 35,000g for 30mins to remove insoluble material. The retinal extract was sterilized by passing it through an 0.2μ filter (ICN/Flow) and stored at -20°C in 0.5ml aliquots. Each batch of extract was assayed for bFGF content in the RIA.

Heparin-Sepharose chromatography

A column containing heparin-Sepharose beads was prepared as described in Section 2.2.2. 30ml of retinal extract containing 4.2 pM of bFGF was applied to the column and the column washed with 50ml of 0.05M Tris/HCl containing 0.1M NaCl. A gradient of NaCl from 0.1-3M was run through the column at 40ml per hour and sixty 0.75ml fractions collected.

Dialysis of eluate from the heparin-Sepharose column

The fractions were pooled in groups of 6 and were then dialysed overnight at 4°C in Visking dialysis tubing (M.W. cut-off = 12,000 daltons) (BDH, Dagenham, U.K.) against 0.05M Tris/HCl pH 7.4. This was to remove NaCl which would interfere in the assays. 20ml 0.05M Tris/HCl pH 7.4 were also dialysed to provide buffer for making up the standards in the RIA so that these could be incubated in the same buffer solution as the samples.

Assay of fractions

After dialysis, 100μl of each fraction was assayed in the RIA in duplicate. The ability of the fractions to stimulate BREC growth was estimated by adding 200μl of each fraction to cells growing in culture as described in Section 2.6.1.

The above experiment was repeated using the same methods except that the 30ml of retinal extract was from a separate batch and contained 4.5 pM bFGF and sixty 1ml fractions were collected from the column. The column was then washed with 20ml of 0.05M Tris/HCl + 4.0M NaCl which were also collected in 1ml fractions. Fractions were pooled in groups of 4 and in addition to dialysis and RIA, the protein content was measured.

Protein determination

The protein content of each fraction was measured using a modified method of Lowry et al., (1951). The following reagents were prepared,

1. Alkaline tartrate reagent 2g Na_2CO_3 and 0.05g K-Na-tartrate in 100ml of 0.1N NaOH
2. 0.1% Copper sulphate reagent 0.05g $CuSO_4$ in 50ml distilled H_2O
3. Folin's reagent (BDH) diluted 1:3 with H_2O - Solution B
4. 90ml of alkaline tartrate reagent were mixed with 10ml $CuSO_4$ just before use - Solution A

Standards were prepared of BSA (0-100 μgml^{-1}) in 0.9% saline. 5ml of solution A was added to 1ml of column fraction or standard in glass test tubes, the tubes were vortexed and allowed to stand for 10 mins at room temperature. 0.5ml of solution B was then added to each tube, the contents mixed and after 30mins at room temperature, the optical density was measured at 750nm using a spectrophotometer (UV160, Shimadzu, Kyoto, Japan).

2.2.5 Affinity chromatography of cell conditioned medium

A preliminary experiment was carried out to determine whether bFGF immunoreactivity measured in BREC conditioned medium could be absorbed onto heparin-Sepharose and eluted with 2M NaCl. Two glass pasteur pipettes were packed with swollen heparin-Sepharose beads and prepared as described in Section 2.2.2. 5ml of bovine retinal endothelial cell (BREC) conditioned medium prepared as described in Section 3.2.5 was run through one column and 5ml of unconditioned culture medium through the other. The columns were washed with 10ml of 0.05M Tris/HCl containing 0.15M NaCl and then 0.05M Tris/HCl containing 2.0M NaCl was run onto the column and 0.5ml fractions collected. These fractions were dialysed overnight at 4°C against 0.05M Tris/HCl pH 7.4 along with 0.05M Tris/HCl containing 0.15M NaCl which was used to prepare the standards for the RIA. 100μl of the dialysed fractions was assayed in duplicate in the RIA.

Subsequently a more detailed examination of the heparin-binding proteins in BREC conditioned medium was made. The size of the column and the volume of the eluting gradient were both kept smaller than for the previous heparin-Sepharose chromatography of rbFGF and retinal extracts. This was because of the relatively low concentration of bFGF in retinal endothelial cell conditioned medium and the need to minimize dilution upon elution. In addition 0.01% w/v 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulphonic acid (CHAPS) (Sigma) was added to each fraction before dialysis in order to prevent loss of bFGF (Matuo et al., 1988) due to non-specific adsorption of bFGF to dialysis tubing and

collecting tubes.

A glass column, 1.0 x 6.0cm was packed with swollen heparin-Sepharose beads prepared as in Section 2.2.1. 12ml of BREC conditioned medium containing4.7 pmol bFGF (measured by RIA, was run onto the column which was then washed with 30ml 0.05M Tris/HCl pH 7.4. Heparin-binding factors were eluted from the column with a gradient of NaCl 0.15-3M in 0.05M Tris/HCl at a flow rate of 10 $mlhr^{-1}$. A total of twenty 1.5ml fractions were collected which were treated by the addition of 1.5μl CHAPS per tube to 0.01% and then dialysed overnight as described above. 100μl of the dialysed fractions were assayed in the RIA in duplicate.

2.3 ELISA for bFGF

Principle

Sandwich ELISA assays are immunometric assays where a binding compound, usually an antibody, is linked to the wells of a microtitre plate. After incubation with the sample or standard a second antibody coupled to an enzyme label, is added and the amount of bound label measured is directly proportional to the amount of analyte in the sample. Often an indirect method of detection is used where a third antibody directed against the immunoglobulin species of the second antibody is coupled to the label. In these assays, the antibody is in excess, this can lead to very sensitive assays dependent on the avidity of the antibody used and also to short incubation times.

An ELISA based on the affinity of bFGF for heparin was developed using modifications of the method of Sato et al., (1989a). Polyclonal bFGF(1-24) antiserum was used as the second antibody and goat anti-rabbit conjugated to alkaline phosphatase (conjugate) was used as the label. Detection of the bound label was carried out using an enzyme amplification technique (Self, 1985).

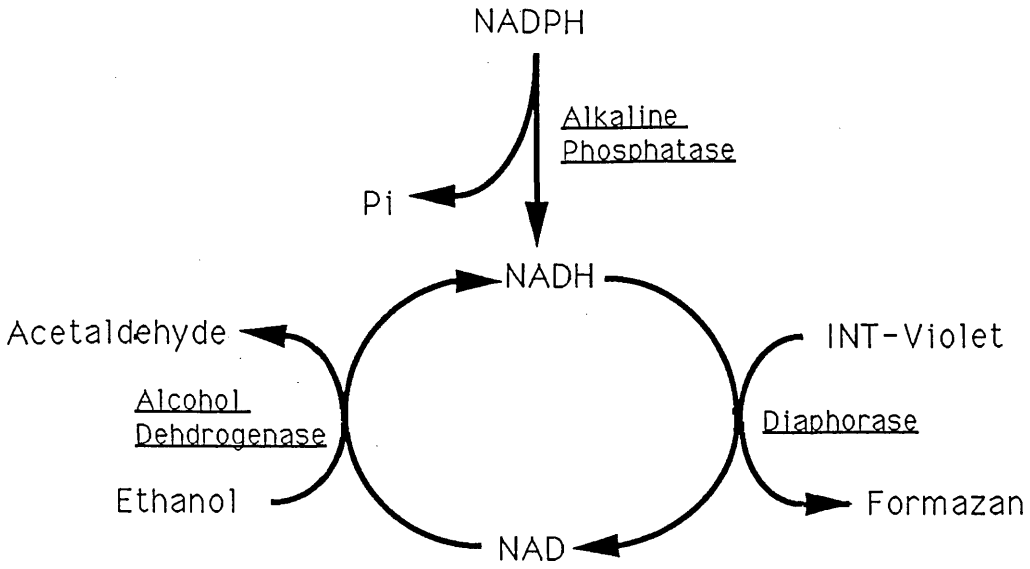


Fig. 2.1 Amplification reaction for alkaline phosphatase detection

In this reaction, shown in Fig. 2.1 there is an initial incubation with the substrate NADPH which alkaline phosphatase dephosphorylates to NADH. Amplifying reagent is then added. This consists of the enzyme diaphorase which links the oxidation of NADH to the conversion of the colourless compound INT-Violet to the coloured formazan product. The enzyme alcohol dehydrogenase then recycles NAD to NADH by coupling it to the oxidation of ethanol to acetaldehyde. This process yields many molecules of coloured dye for every molecule of NADH formed thereby amplifying the action of the enzyme label. Formazan dye is a red coloured product that can be quantified using a spectrophotometer at 490 nm. The sensitivity and dynamic range of the assay can be increased by the kinetic measurement of colour development (Stanley et al., 1985).

Subsequently an ELISA was developed for bFGF using 96-well microtitre plates coated with monoclonal antibodies raised against recombinant bFGF which were the gift of Dr. T. Reilly, Du Pont, Delaware, U.S.A. This assay also utilised the bFGF(1-24) antibody as the second antibody with the label and detection method as described above.

2.3.1 Heparin based ELISA

Plate preparation

Polycarbonate 96-well ELISA plates (Alpha laboratories, Eastleigh, U.K.) were cleaned by washing once with saline containing 0.1% Tween 20, once with distilled water and once with ethanol folowed by drying. Plates were stored in ethanol prior to use.

Coating the plates with heparin

Plates were first coated with poly-L-lysine (Sigma) to provide an effective substrate for heparin binding. 100μl of poly-L-lysine (100 μgml^{-1}) in distilled water was added to each well and the plates incubated overnight at 4°C. Remaining solution was removed and 100μl of heparin (Sigma) (1mg/ml) was added to each well. After an overnight incubation at 4°C the wells were washed with 0.01M phosphate buffer (pH 7.4) containing 0.5M NaCl (PBS) and covered with 1% BSA in PBS containing 0.04% sodium azide. The plates were sealed with a plate sealer (ICN/Flow) and stored at 4°C until use.

Assay buffer

The assay buffer was 25mm Tris containing 0.15M NaCl and 0.1% BSA. All standards and reagents were diltued in this except where indicated.

Optimization of antibody, conjugate, substrate and amplifer concentrations

Standard curves were constructed using a range of bFGF concentrations form 0 to 6100pM. These were incubated in heparin coated plates and the assay developed with bFGF1-24 antibody at concentrations of 1:100,000, 1:20,000 and bFGF1-10 antibody at concentrations of 1:1000, 1:2000 and a range of conjugate concentrations, 1:250, 1:500, 1:1000. Substrate

and amplifier were used both neat and diluted 1:1 with distilled water and both end point, (3 and 5 mins.), and kinetic measurements of absorance at 490nm were carried out using a V-Max 96 well spectrophotometer (Molecular Devices, California, U.S.A) linked to an Archimedes mircomputer (Acorn Computers Ltd., Cambridge, U.K.). Data reduction was carried out using Novoclone software (Novobiolabs, Cambridge, U.K.).

Optimized assay procedure

100 µl of the standards (0-6100pM) or samples were incubated at 4°C in duplicate in heparin coated 96 well ELISA plates covered with plate sealers. The wells were then washed with assay bufer and 100µl of bFGF 1-24 antibody (1:20,000) was added to each well and the plates incubated overnight a 4°C. The plates were again washed with assay buffer and 100µl conjugate (1:1000) added to each well followed by a further overnight incubation at 4°C.

Colour development

All reagents used were Ampak, (Novobiolabs). The wells were washed 4 times with washing buffer diluted 1:12 and then 100µl of substrate diluted 1:1 with distilled water was added for 5 minutes. Amplifier diluted 1:1 with distilled water was added, 100µl/well, and the plate immediately loaded into the Vmax and read kineticaly after an initial shake with 21 determinations, one every 20 seconds.

2.3.2 Monoclonal antibody based ELISA

In order to overcome the possible interference of heparin-binding substances other than bFGF present in the samples in the heparin-based ELISA, a sandwich assay was developed using monoclonal antibodies against bFGF to coat 96-well plates.

Coating plates with monoclonal antibody

Four monoclonal antibodies raised against rbFGF and purified by affinity chromatography (Reilly et al., 1989) were assessed for their ability to bind rbFGF in a sandwich ELISA. These antibodies were designated by the following codes, AF11, DE6, DG2 and FE8 and had a protein concentration of 0.78, 0.9, 1.06 and 0.72 $mgml^{-1}$ respectively. They were stored in aliquots containing 12.5μg of protein at -20°C until use. Initially, each of the four antibodies was diluted to 5μgml^{-1} with 0.01M sodium phosphate buffer pH 7.4 containing 0.14M NaCl (PBS) and 100μl was added to each well of a 96-well ELISA plate (Greiner Labortechnik, Dursley, U.K.). The antibody was allowed to coat the plate for 18 hrs. at 4°C. There was no success with this method so the antibody concentration was increased to 20μgml^{-1} diluted in bicarbonate buffer (1.59g Na_2CO_3 and 2.93g $NaHCO_3$ per litre) pH 9.0. 100μl was added to each well and incubated as above.

Assay procedure

A wide range of buffers, blocking agents, antibody dilutions and assay conditions was tried as described under assay optimization. The basic outline of the assay was as follows :- 96-well ELISA plates were coated with monoclonal antibody after which the antibodies were removed and the wells washed. Unreacted sites were blocked with a blocking agent and this was washed away before the wells were incubated with sample or standard rbFGF. The sample was then washed away and the wells incubated with FGF 1-24 antibody followed by GAR antibody coupled to alkaline phosphatase. Colour development was carried out as described in Section 2.3.1. and the plates read kinetically.

Assay optimization

It was decided to carry out all coating of plates with monoclonal antibodies in bicarbonate buffer as above. The initial experiment used 1% BSA as the blocking agent, 0.05M

NaPhosphate buffer pH 7.2 and 0.02% gelatin as the assay buffer and rbFGF standards (0-512 pM) made up in serum-free cell culture medium (see Section 2.5.2). Each attempt produced a high background with indistinguishable standard responses. To determine whether monoclonal antibody was coating the plate and binding rbFGF, each monoclonal antibody was used to coat a polyvinyl chloride 96-well plate. Some wells were coated with a 1:10 or a 1:25 dilution of non-immune mouse serum (NIMS) (Biogenesis) in bicarbonate buffer or with bicarbonate buffer alone as a control. The wells were then washed with bicarbonate buffer and blocked with bicarbonate buffer containing 3% BSA for 18hrs at room temperature. The wells were washed again with bicarbonate buffer followed by the addition of 100μl ^{125}I-rbFGF (100,000 cpmml^{-1}), iodinated as described in Section 2.1.3, to each well in FGF 1-24 buffer and 0.2% gelatin. The plates were incubated overnight at 4°C, washed with bicarbonate buffer and each well was cut from the plate and counted in a gamma-counter for 1 minute.

The use of BSA as the blocking agent produced uniformly high backgrounds due to antibodies against this protein in the anti-bFGF(1-24) antiserum. So, 5% w/v Marvel low fat milk powder was also tried along with the effect of preincubating the bFGF 1-24 antibody with BSA in order to bind antibodies directed against this protein. 5mg BSA were added per ml of 1-24 antibody (1:5000)in FGF 1-24 buffer. The incubation times for the reaction of 1-24 antibody and the conjugate were both reduced from 18hrs at 4°C to 2hrs at room temperature.

Optimized assay procdure

96-well ELISA plates were coated with one of the four monoclonal antibodies by the addition of 100μl per well (20μgml^{-1}) in bicarbonate buffer pH 9.0 for 18hrs at 4°C. The wells were washed with 300μl per well PBS and 100μl 5% Marvel in PBS was added to each well for 5hrs. The wells were then washed twice with 300μl FGF 1-24 buffer containing 0.2% gelatin followed by addition of 100μl of samples or

standards. The plates were incubated overnight at room temperature. They were then washed twice with 300μl FGF buffer and 0.2% gelatin followed by the blocking of non-specific binding sites by the addition of 100μl per well FGF 1-24 antibody (1:5000), which had been preincubated with 5mgml^{-1} BSA in FGF buffer for 2hrs at room temperature. The plates were incubated for 2hrs at room temperature, washed twice with FGF buffer and 0.2% gelatin and 100μl GAR-alkaline phosphatase (1:1000) in FGF buffer and 0.2% gelatin was added to each well for a further 2hrs at room temperature. Colour development was carried out as for the heparin-based ELISA except that both the substrate and amplifier were used neat. The plates were read kinetically at 490nm. Samples and standards were diluted 1:1 with FGF buffer containing 0.4% gelatin.

2.3.3 Immunoblotting

Recombinant bFGF and purified aFGF were diluted to give a range of concentrations from 0.02-20 pmolμl^{-1}. 1μl of each was spotted directly onto nitrocellulose membranes (Anderman, Kingston-Upon-Thames, U.K.) alongside 1μl aliquots of medium which had been conditioned by BREC and BRP for 48 hours and unconditioned medium as a control. After allowing the spots to dry each membrane was wetted by floating on a solution containing 10mM Tris-HCl pH 8.0, 150mM NaCl and 0.05% v/v Tween 20 (Sigma) (TBST). The membranes were incubated in TBST and 1% w/v BSA for 30 mins in order to block non-specific binding sites. The membranes were then incubated with either bFGF monoclonal antibody AF11 (20μgml^{-1}) or with anti-aFGF antibody (1:4000) (British Biotechnology, Oxford, U.K.). After 30 minutes each membrane was washed 3 times for 10 minutes each with TBST. The membrane incubated with the monoclonal antibody was incubated with GAM-alkaline phosphatase (1:1000) (Sigma) and the other membrane with GAR-alkaline phosphatase (1:7500) (Promega, Southampton, U.K.) for 30 mins. After a further 3 washes with TBST the membranes were blotted on filter paper and the signal was developed in 100 mM Tris-HCl pH 9.5 containing 100mM NaCl, 5mM $MgCl_2$, 66μl

of NBT substrate and 33μl BCIP substrate (Promega). After colour development the membranes were washed in distilled water.

2.4 Radioimmunoassay for IGF-1

Principle

Insulin-like growth factor 1 circulates bound to a binding protein and cells in culture have also been shown to secrete an IGF-1 binding protein (Bar et al., 1987) . IGF-1 must therefore be removed from its binding protein before radioimmunoassay because the assays available cannot detect the free hormone. Removal of binding protein was achieved by incubating the sample with acid/ethanol to both break the bonds between IGF-1 and the binding protein and precipitate the latter out of solution. The acid pH of the resulting solution was neutralised by the addition of 2-amino-2-hydroxymethylpropane-1,3-diol (Tris) before assay. The RIA used a double antibody precipitation method with a polyclonal IGF-1 antibody raised in rabbits and GAR as the precipitating antibody.

Sample preparation

200μl of sample was mixed with 800μl of acid/ethanol (12.5% v/v 2M HCl/87.5% v/v ethanol) and allowed to stand at room temperature for 30mins during which time a precipitate formed. The tubes were spun at 1400g for 10mins and 500μl of the supernatant was removed from the top of each tube and neutralized by the addition of 200μl of Tris base (0.855M, pH 10.8). The tubes could be kept at 4°C for up to 1 week before assay during which time a further precipitate formed in samples containing serum.

Assay procedure

Recombinant insulin-like growth factor 1 (Amersham) was dissolved in 286μl Tris base and 714μl of acid/ethanol was

then added. The solution was then double diluted in neutralized acid/ethanol to produce a range of concentrations from 0.22-229 nM. Acid/ethanol treated samples were centrifuged at 1400g for 20mins before use. Assay buffer was 0.3M NaPhosphate buffer pH 7.4 containing 0.01M EDTA and 0.2 gl^{-1} protamine sulphate. 2.5g of BSA (RIA grade, Sigma) was added just before use. 20μl of the standards or sample were incubated with 180μl of assay buffer, 100μl of ^{125}I-IGF-1 (74 TBq/mmol) (Amersham) diluted to give 100,000 $cpmml^{-1}$, 100μl NIRS (1:800) and 100μl anti-IGF-1 antibody (1:3600) (UB 486, the gift of Drs. L. Underwood and J.J. Van Wyck, University of North Carolina, Chapel Hill, NC). NSB tubes substituted 100μl of assay buffer for the antibody. The tubes were incubated for 48hrs at 4°C and 100μl of GARS (1:80) was added to each tube, the tubes were incubated for a further 30mins at 4°C and 1ml of ice cold, 6% w/v polyethelyene glycol was added to each tube. The tubes were immediately vortexed and then centrifuged at 1400g for 20mins to seperate bound from free label. Free label was removed by aspiration and the bound fraction counted with a gamma-counter. The assay had a sensitivity of 0.3nM and the inter-assay variation was 8% at 14nM.

2.5 BREC and BRP culture

Principle

Bovine retinal endothelial cells and retinal intramural pericytes were selectively cultured from neural retinae isolated from fresh bovine eyes obtained from a slaughter house. The retinae were homogenized and passed through syringe filters containing nylon filter screens. The retinal vessels were trapped on the nylon screens, scraped and washed into culture medium. The vessels were treated with protease enzymes to break them into smaller fragments, remove surrounding neural tissue and digest the ECM around these vessel fragments. The enzymes were washed away and the vessel fragments suspended in cell culture medium and plated into tissue culture flasks. Several medium formulations and

supplements were used and their usefulness will be discussed. The medium was formulated in order to provide the appropriate nutrients and growth factors for the particular cell type. In addition a variety of matrix components have been used to coat the tissue culture flasks and tested for their ability to support retinal endothelial cell growth. These matrix components simulate the ECM on which the cells are situated 'in vivo'. The selective culture of either retinal pericytes or retinal endothelial cells from the same vessel fragments relied on two factors. First the growth medium and the substrates were selected to promote the growth of the specific cell type required and secondly, the different rate of growth of the cell type and their different adherence to the substrate were used to purify the cells when they were passaged.

Cells in culture grow and proliferate and will eventually fill the available space for growth, this stage is called confluence. Confluent or pre-confluent cells were passaged which involved removing the cells from their flask and seeding them into new flasks at a lower density allowing them to grow and proliferate further. Trypsin was used to break the attachment of the cells to the substrate. A single cell suspension was then produced by mechanical disaggregation and the cells diluted to the required density and reseeded into new tissue culture flasks.

All isolation and passaging procedures were performed in a Gelaire BSB 4A, Class II microbiological cabinet (ICN/Flow). Antibiotics were included in the solutions for the isolation procedure, the initial 18hrs of cell culture and in the wash solutions to suppress bacterial growth. We did not include antibiotics in the growth media because of their possible interference in experimental procedures and because their use could mask a low level infection which would also interfere with cell function.

BREC and BRP were characterized by their expression of specific antigens which were determined immunocytochemically.

2.5.1 Isolation and preparation of retinal vessels

The isolation and homogenization of the neural retina was a standard procedure. All instruments were sterilized by soaking in 70% v/v ethanol and washed in sterile distilled H_2O before use. The homogeniser and the filter units were sterilized by autoclaving at 120°C and 15 psi for 30 mins. Eyes were generally processed in batches of 20 although this could be altered to suit requirements. Bovine eyes from freshly slaughtered bullocks were obtained from the slaughter house (Ziff Meats, London, U.K.). These eyes could be stored for at least 28hrs at 4°C before use without loss of isolated cell viability although the neural retina was more easily damaged and more difficult to remove at this time. Most of the surrounding fat and muscle tissue was dissected away from around the globes and these were then placed in a 10% w/v Povidone/iodine solution (Betadine, Napp Laboratories, Cambridge, U.K.) to sterilize the outer tissue. The globes were washed in sterile distilled H_2O and placed on sterile tissue on a specially constructed stand which had a detachable concave wooden insert on top (R.P.M.S workshops). This provided a stable seat for the globe on which it could be rotated and dissected. The globe was then bisected at a point just behind the internal position of the lens using a backless strip of razor blade (Sabre Int., Reading, U.K.) held in a pair of artery forceps. A hinge of scleral tissue between the front and back half of the globe was left intact. A pair of curved, blunt dissecting scissors were pushed between the neural retina and the pigment epithelial layer and the vitreous pushed forward so that it became detached from the retina. The neural retina was pulled away from the underlying pigment epithelium and then cut at the optic nerve. It was removed with a pair of dog-toothed forceps and placed in a sterile petri-dish containing 20ml of Minimal Essential Medium containing 25mM 4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) and 1% v/v penicillin/streptomycin (10,000 U penicillin, 10,000 µg/ml streptomycin) (Gibco, Paisley, U.K.) (MEM). 10 retina were placed in 20ml of MEM and the retina agitated to dislodge any

adherent pigment epithelium. They were then homogenised in 10ml MEM in a Teflon /glass homogenizer (Jencons, Leighton Buzzard, U.K.) by manually rotating the plunger up and down for 1.5mins to produce a microvessel suspension.

Two different methods were tried for the succeeding steps in the isolation and preparation of microvessel fragments. The first is based on the method of Wong et al., (1987). The retinal homogenate was spun at 300g for 10 minutes, placed in 10ml PBSA and layered onto a sterile 83μ nylon mesh (Henry Simon Ltd., Stockport, U.K.). The homogenate was stirred through the mesh and the trapped vessels were washed off into 5ml of a solution containing the following enzymes:- 70 μgml^{-1} deoxyribonuclease 1, 0.35 $mgml^{-1}$ pronase and 0.7 $mgml^{-1}$ collagenase (all Sigma) in PBSA sterilized through an 0.2μ filter. This mixture was placed in a sterile petri dish on a plate shaker (Chemlab Ins., Hornchurch, U.K.) in an incubator (LTE, Oldham, U.K.), at 37°C for 15 mins when isolating pericytes and 20mins when isolating endothelial cells. The digested vessel fragments were then placed onto a 53μ nylon mesh and stirred through as above. The trapped vessels were then washed into 10ml of of MEM and this mixture divided between several sterile centrifuge tubes. The small volume of liquid in each tube prevented the vessels from sticking to the sides. The tubes were centrifuged at 300g for 5mins, the supernatant poured off and the vessels resuspended in MEM. The vessels were again spun at 300g for 5mins, the supernatant poured off and the vessels suspended in 1ml of cell growth medium (see Section 2.5.2 below).

The second method for microvessel preparation was based on that of McIntosh et al., (1988) and was used routinely. The homogenized retinae were divided into 4 aliquots and each was added to 14ml of MEM in a 30ml universal container (Sterilin, Bargoed, U.K.) and mixed by pipetting. Each mixture was taken up into a 10ml syringe and filtered through a metal filter casing (Millipore, Watford, U.K.) containing an 85μ nylon filter and washed with a further 10ml of MEM. The nylon filter discs were removed using a pair of 21 gauge needles and the trapped microvessels scraped off using a sterile cell scraper (Costar, High Wycombe, U.K.) and washed

into MEM, 2.5ml per filter, containing 2mgml^{-1} collagenase/dispase (Boehringer Mannheim, Lewes, U.K.). The washes were pooled and placed in a 25 cm^2 tissue culture flask. The flask was gassed with 95% air:5% CO_2 and sealed. It was then placed in an IR1500 incubator (ICN/Flow) and tipped end on end for 90mins at 37°C on a specially designed shaker (R.P.M.S. workshops) which produced very little heat during operation and prevented a temperature rise in the incubator. The vessel digest was divided into 4 and each aliquot was placed in 20ml Hank's Balanced Salt solution (HBSS) (Gibco) (10% v/v HBSS x10, 10mM HEPES, 27mM $NaHCO_3$, 100 IUml^{-1} penicillin and 100 μgml^{-1} streptomycin) at 37°C to dilute the enzyme and vortexed very briefly. The vessel fragments were centrifuged at 230g for 6mins and the supernatant discarded. The vessel fragments were suspended in cell culture medium and placed into tissue culture flasks as described in Section 2.5.2.

2.5.2 Growth and subculture of cells

Bovine retinal pericytes

The standard growth medium for BRP was Dulbecco's Modified Eagle's Medium with 20% v/v foetal calf serum. The original formulation was 8% v/v DMEM x10, 10mM HEPES, 21mM $NaHCO_3$, 10% v/v Tryptose Phosphate Broth (TPB) (Gibco), 0.45mgml^{-1} glutamine and 0.55mM NaOH to pH 7.4. Subsequently, DMEM x1 was used containing 20% FCS and 10% Tryptose phosphate broth. For the initial seeding 100 iuml^{-1} penicillin and 100μgml^{-1} streptomycin were added. The microvessel suspension from 20 eyes was split between six uncoated 75 cm^2 tissue culture flasks each containing 10ml DMEM with 20% FCS. The vessel fragments were allowed to attach overnight at 37°C in an atmosphere of 95% air:5% CO_2 in sealed flasks. The pH was maintained at pH 7.4 by the buffering capacity of the medium at this concentration of CO_2. The attached vessels were washed with 10ml HBSS and 10ml of fresh DMEM containing 20% FCS was added and the flasks allowed to equilibrate at 5% CO_2 before they were sealed.

Bovine retinal endothelial cells

Two methods were tried, the first based on that of Wong et al., (1987) and the second on that of McIntosh et al., (1988). The first method used gelatin as the growth substrate. Porcine gelatin (200 bloom) 1% w/v was dissolved in double distilled water by heating to 70°C and was sterilized by filtration through an 0.2μ filter. 1.5ml was placed in every 25 cm^2 flask which were incubated at 37°C for 4hrs. The excess solution was removed and the remaining solution allowed to dry out overnight in the Class II cabinet. Coated flasks could be stored at 4°C until used. The medium used for endothelial cell growth was DMEM containing 7.5% Platelet Poor Serum (PPS) which was mixed 1:1 with pericyte conditioned medium. Retinal extract, 20μlml^{-1} (prepared as described in Section 2.2.4.), 90 μgml^{-1} heparin and 0.2 μgml^{-1} insulin were added to the medium before use. PPS was prepared by centrifuging sterile citrated human pooled plasma at 35,000g for 30mins, heat inactivating the plasma at 56°C for 35mins and again centifuging at 35,000g for 30mins. The plasma was then dialysed overnight against PBSA which produced fibrin clots in the plasma and the serum was centrifuged at 35,000g for 30 mins to remove the clot and was steilized by filtration through an 0.2μ filter. The serum was stored frozen at -20°C in 10ml aliquots prior to use. Pericyte conditioned medium was prepared by growing BRP till just prior to confluence. The cells were then washed with HBSS and 10ml of DMEM with 7.5% PPS was added per 75 cm^2 flask. After 48hrs the medium was removed, centrifuged at 925g for 10mins to remove debris and stored at -20°C before use.

The second method used human plasma fibronectin, (initially obtained from Sigma and then The New York Blood Centre, New York, U.S.A.) as the growth substrate. The fibronectin was diluted in sterile PBSA and stored in aliquots at -20°C until used. To coat the flasks 0.03ml of fibronectin (50μgml^{-1}) was added per cm^2 of flask area and the flask shaken so that the solution thoroughly coated the bottom of the flask. Flasks were placed at 37°C for at least

1hr before use so that sufficient fibronectin was adsorbed by the plastic surface of the flask. The growth medium used in the second method was initially Glasgow's Modified Eagle's Medium containing 10% v/v TPB and 10% v/v Plasma Derived Serum (PDS) but was superceded by 8% v/v DMEM x10 containing 10% TPB and 10% PDS and finally DMEM x1 with 10% TPB and 10% PDS. PDS was prepared by dialysing citrated pooled human plasma against 3 changes of buffer solution at 4°C over 3 days. The buffer solution contained the following components per litre:- 8g NaCl, 0.2g KCl, 1.78g $Na_2HPO_4.2H_2O$, 0.132g $CaCl_2.2H_2O$, 0.2g KH_2PO_4 and 0.1g $MgCl_2.6H_2O$. For plating, microvessels were suspended in DMEM containing 10% PDS and penicillin/streptomycin (40ml per 20 retinae) and 10ml placed in each of four 75 cm^3 flasks which were allowed to equilibrate at 5% CO_2 and 37°C before being sealed. After 18hrs they were washed with HBSS and fresh DMEM + 10% PDS added.

Trypsinization and purification

BREC and BRP were allowed to grow until they were approaching confluence (sub-confluent), about 4 days for BREC and 11 days for BRP. Medium was not changed on the BREC but was changed every 3 days on the BRP. At this stage the cells were passaged and contaminating BREC in pericyte cultures and BRP in endothelial cultures were removed as follows. For BRP, the monolayer was washed once with HBSS and then 10ml HBSS was added for 10mins at 37°C. The HBSS was removed and 4ml trypsin (ICN/Flow) diluted 1:10 with HBSS to 0.25% v/v was added per 75 cm^2 flask at 37°C. The cells were placed in the incubator for 2mins., viewed down a phase contrast microscope, (Olympus, London, U.K.) and when the loosely adhering contaminating retinal endothelial cells had detached, the flask was banged against the palm of the hand to dislodge the cells which were washed off with HBSS and removed. This process, called differential trypsinization, increased the purity of the BRP. Fresh 0.25% trypsin was added to the flasks and they were incubated at 37°C for 15mins or until the cells had visibly detached. 14ml DMEM

with 20% calf serum (CS) (Gibco), prepared as the DMEM containing 20% FCS, was added to each flask to neutralize the trypsin and the mixture was placed in 30ml universal containers and centrifuged at 230g for 6mins. The supernatant was removed and DMEM containing 20% FCS was added and pipetted up and down to break up the cell pellet. The cell suspension was diluted with DMEM containing 20% FCS and seeded into 75 cm^2 flasks so that cells from 1 confluent flask were added to 3 new flasks (1:3 split). They were allowed to equilibrate at 95% air:5% CO_2 before being sealed. For BREC, the sub confluent cell monolayer was washed with HBSS and 10ml of HBSS was added for 10mins at 37°C. This was removed and 4ml of 0.25% trypsin was added and the flasks incubated at 37°C for 1min. The cells were viewed down the microscope and when they had rounded up the flask was banged against the palm of the hand to dislodge the cells and 14ml DMEM containing 20% CS was added. The cells were then removed and placed in 30ml universal containers. The contaminating BRP remained adherent to the flask and this resulted in a purification of the BREC cultures. The cells were centrifuged at 230g for 6mins, the supernatant removed and 5 ml of DMEM containing 10% PDS was added to each container. The cells were disaggregated mechanically by passing them up and down twice through a 21 gauge needle and were centrifuged at 230g for 6mins. Fresh DMEM with 10% PDS was then added and the cells pipetted and diluted. They were seeded into 75cm^2 flasks at a 1:3 split, equilibrated with 95% air:5% CO_2 and sealed.

2.5.3 Characterisation of BREC and BRP by immunoflourescent staining

Principle

The definitive identification of a particular cell type and the homogeneity of a cell culture depends on the detection of specific markers expressed by that cell type. Antibodies directed against the marker are incubated with cultured cells, non-immune serum incubated cells serving as

a control, and bound antibody is visualized with a second antibody directed against the immunoglobulin-G of the species in which the first antibody was raised coupled to a chromogenic or fluorescent label. Several specific markers exist for retinal endothelial cells, we chose Von Willebrand's Factor, also known as Factor VIII related antigen. There is only one specific marker known for BRP to which antibodies have been raised. This ganglioside antigen is called 3G5 (Nayak et al., 1988).

Characterization of BREC

Sub confluent BREC were trypsinized and seeded into each well of an 8-well Labtek slide (ICN/Flow). which had been previously coated with fibronectin. BRP cells were seeded into non coated Labtek slides as a control. These sterile glass microscope slides have detachable chambers, fixed to the top of the slide by a gasket, in which cells can be grown. All volumes of incubating or wash solutions were 200μl. At confluence the medium was removed and each well washed 3x with MEM. The cells were fixed by the addition of of ice cold methanol for 45 seconds followed by 3 further washes with MEM. Non-specific binding sites were blocked by the addition of a 1:20 dilution of NIRS in MEM for 5 mins and the cells were washed once with MEM. Wells were then incubated with either anti-Factor VIII related antigen (Dako, High Wycombe, U.K.) 1:100 or 1:200 in MEM or NIRS (1:20) for 60mins at room temperature. The antibodies were removed and the cells washed with MEM 3 times. Non-immune goat serum (NIGS) 1:20 in MEM was added for 5mins to block non-specific binding sites for the second antibody, the wells washed once with MEM and the cells incubated with a 1:50 dilution of fluorescein conjugated GAR in MEM (Sigma) for 30mins at room temperature. The cells were again washed 3x with MEM and the chamber wells and the gasket removed from the slide taking care not to dislodge the cells. The slide was placed in PBSA for 30 seconds before mounting with a coverslip on a drop of Hydromount (National Diagnostics Inc., Manville, U.S.A). The slides were kept in the dark to prevent bleaching of the

flourescein label. Slides were examined for fluorescent staining with a Leitz Dialux 22 fluorescent microscope (Leica Instruments, Nussloch, Germany).

Bovine retinal pericytes

BRP and BREC were grown in 8-well Labtek slides and when subconfluent, the medium was removed and the cells washed 3 times with PBSA containing 1% w/v BSA. They were then incubated for 1hr at room temperature with either a 1:100 dilution of 3G5 antibody or a 1:20 dilution of NIMS as a control, both dilutions were made in PBSA. The cells were washed 3x with PBSA containing 0.1% w/v BSA and incubated for 30mins at room temperature with a 1:20 dilution of fluorescein conjugated GAM (Sigma). After washing 3x with PBSA containing 0.1% w/v BSA the chamber wells and gasket were removed and the cover slip mounted as above. Slides were examined as described above.

2.5.4 Preparation and growth of cells on Millicell inserts

Principle

Millicell cell culture inserts are culture vessels containing either a cellulose ester membrane with an 0.45μm pore size (HA) or a Biopore membrane with an 0.4μm pore size (CM) (MIllipore, Watford, U.K.). They are available in two different sizes (12 and 30mm diameter) which can be placed in the wells of either a 6-well or a 24-well cell culture plate. Both membrane types were tried, the CM insert was transluscent and allowed the cells to be viewed down the microscope. The inserts allow cells to be grown on a membrane which is permeable to ions, nutrients, proteins and other molecules and were used in an attempt to measure polarized bFGF release from the apical and the basolateral surface of the cell.

Preparing the inserts

30mm inserts were placed in each well of a 6-well cell culture plate and 700μl of fibronectin (50μgml^{-1}) was added to each insert. The solution was allowed to airdry overnight in a Class II laminar flow hood. 1.0ml of DMEM + 10% PDS was added to each well of a seperate 6-well plate and a coated insert placed into each well ensuring that no air was trapped beneath and the membrane was thoroughly wetted.

Seeding and growing BREC

A subconfluent monolayer of BREC was trypsinized and a single cell suspension of BREC produced (Section 2.5.2). The cells were counted using a Coulter counter (Section 2.6.3) and 100,000 cells per well in 0.5ml was added to the inserts. A lid was placed on the plate and these were placed in polycarbonate boxes (2 per box) alongside 25ml glass beakers containing water for evapo ration. Sterile distilled H_2O containing 1% v/v penicillin/streptomycin and 0.2% Fungizone (250 μg/ml Amphotericin B) (Gibco) and was used to prevent evaporation of culture medium from the culture wells. The box was allowed to equilibrate at 5% CO_2 and the lids then closed.

2.5.5 Cryopreservation of cells

Principle

Cryopreservation is necessary to provide batches of cells of the same passage number from separate isolations for experiments and to maintain stocks of cell lines obtained from external sources. The cells must be kept frozen at the very low temperature of liquid nitrogen (-196°C) or, for short periods of time, its vapour phase (-130 to -165°C) to maintain viability. The cells are frozen at a controlled rate to prevent water being lost too slowly from the cell by osmosis if the cells are cooled to slowly, or the cells being unable to lose water if they are frozen too quickly. In both

cases ice crystals will form intracellularly resulting in cell damage. A cryopreservant must also be added to the solution in which the cells are frozen. This lowers the optimum cooling rate and allows the cells to reach a lower temperature before freezing, allowing all the water to leave the cell.

Cell freezing

A sub-confluent 75 cm^2 flask of cells was trypsinized and a single cell suspension produced in 0.5ml of growth medium. The cells were transferred to a 1.8ml cryovial (Greiner Labortechnik) and 0.5 ml of a sterile solution of 20% v/v dimethylsulphoxide (DMSO) in DMEM was added. The vial was wrapped in cotton wool placed in a small cardboard box and left to freeze overnight at -70°C in a low temperature freezer. This ensured that the rate of freezing was about 1°Cmin^{-1} and within the optimum range for cell viability. The cryovials were then placed on a cane and slowly lowered into a flask containing liquid nitrogen which was topped up at regular intervals.

Cell thawing and viability

The cells were thawed rapidly by shaking the vial in a water bath at 37°C until all the ice had melted. The cells were then diluted with 10ml of growth medium and added to a 75 cm^2 flask. After the cells had attached overnight, the monolayer was washed once with HBSS and fresh growth medium added. In order to assess the viability of BREC after cryopreservation, vials of frozen cells were thawed and 20μl of 0.4% w/v Trypan blue (Gibco) was added to 20μl of the cell suspension. Total cells and non-viable cells which had taken up Trypan blue were counted using a haemocytometer as described in Section 2.6.3 and the percentage viability calculated. The use of 50% serum in the freezing mixture and 10% v/v glycerol as an alternative cryopreservant to 10% DMSO were also assessed by freezing cells from the same batch in either of these solutions and testing their seeding ability after a period of

2 months.

2.5.6 Astrocytoma cell culture

Principle

Human astrocytoma cells were used as a control cell in some of the experiments on bFGF production by retinal cells because of their demonstrated production of high levels of this growth factor (Murphy et al., 1988a). They are a tumor cell line and as such can be grown indefinitely with repeated passage. A stock control was however maintained frozen as described in Section 2.5.5 in order to provide fresh cells as a safeguard against phenotypic changes arising in the cells with time.

Culture conditions

Human astrocytoma cells (U-87 MG) (American Type Culture Collection, Rockville, Maryland, U.S.A.) were grown on a tissue culture plastic substrate in DMEM containing 10% FCS. Medium was changed every 3 days and the cells were grown to confluence and passaged by trypsinization as described in Section 2.5.2.

2.6 Bioassay for growth factors

Principle

Bovine retinal endothelial cells were used to test human serum, retinal extracts, cell conditioned medium and heparin-Sepharose column fractions for the presence of growth factors. The ability of purified growth factors to stimulate their proliferation was also assessed. Several methods were used to measure cell growth and proliferation and these are described below.

2.6.1 Coomassie blue

Principle

This method, based on that of Laughton, (1984), relies on the staining of total cell protein with Coomassie blue protein stain in 96-well tissue culture plates. Staining is proportional to cell growth and proliferation and is measured spectrophotometrically at 630nm in each well. In order to eliminate the variation in cell growth between plates, the optical density in test wells is compared to that in control wells in which the cells are grown under standard conditions.

Preparation of 96-well tissue culture plates

50μl of fibronectin (50 $\mu g ml^{-1}$) was added to each of the 60 inner wells of a 96-well plate. 200μl of sterile distilled H_2O was added to each of the 36 outer wells of the plate. This was to prevent evaporation in the wells affecting the concentration of medium and test substances, thereby altering cell growth. The plates were placed in polycarbonate boxes with lids, prepared as described in Section 2.5.4, to prevent evaporation. They were placed in an incubator at 37°C for at least 1hr before use.

Seeding of cells and addition of test agents

Test agents, diluted to give the required concentration when in the final volume in the well (200μl), were added to the inner 60 wells of the plate in 100μl of culture medium alongside control wells containing culture medium alone. At least 10 wells were used per treatment or control. Cell proliferation assays were carried out on 1st or 2nd passage BREC. An optimum cell concentration for seeding cells into 96-well plates was determined by carrying out growth characterization experiments (See Section 5.2.1). Sub-confluent BREC were trypsinized and counted with a haemocytometer as described in Section 2.7.3. They were diluted with DMEM containing 10% PDS to 80,000 $cells ml^{-1}$ and

50μl (4000) cells were added to each well. The plates were then placed in a polycarbonate box as described above, allowed to equilibrate at 5% CO_2 in the incubator and then incubated for a further 48hrs. The appropriate length of incubation with the test agents was also been determined by growth characterization experiments (Section 5.2.1).

Preparation of Coomassie blue

An 0.04% w/v stock solution in 3.5% w/v perchloric acid was prepared by adding 0.4g of Coomassie Brilliant Blue G-250 (Sigma) to a solution containing 58.5ml of 60% w/v perchloric acid and 941.5ml distilled H_2O. The dye was dissolved by placing it in a beaker on a plate mixer overnight at room temperature. The resulting solution was filtered through Whatman No. 1 filter paper (Whatman, Maidstone, U.K.) and was then ready for use. It could be stored for up to 1 month at 4°C in the dark.

Cell staining

Culture medium was removed from each inner well of the 96-well plate using an 8-pronged variable pipette (ICN/Flow) with which all further manipulations of solutions in the wells was carried out. The cells were washed 3 times with PBSA by removing 100μl of medium from the wells, adding 100μl PBSA and then repeating this procedure in order to prevent dislodging cells from the bottom of the wells. 100μl of Coomassie blue dye was then added to each well and left for 5mins. This process fixed the cells. The stain was removed by pouring and shaking and a further 100μl of Coomassie blue dye was added to each well. The plate was covered with a plate sealer and incubated for at least 3hrs at 37°C or 5hrs at room temperature and the optical density was measured at 630nm using a Titertek Multiskan (ICN/Flow). The plate could be stored at room temperature, covered with a plate sealer, in the dark, for up to 5 days before the optical densities were measured.

2.6.2 Methyl-^{3}H thymidine

Principle

This method measures the incorporation of methyl-^{3}H thymidine into the DNA of dividing cells. The cells take up the labelled thymidine from the medium and actively dividing cells incorporate this nucleotide into the newly synthesized strand of DNA. Incorporation is proportional to cell proliferation. The cells were seeded into multiwell plates. Two methods were used. In the first test agents were added 24 hours before the addition of methyl-^{3}H thymidine to the medium. In the second the cells were serum starved after attachment by the addition of serum-free DMEM or DMEM containing 0.1% PDS for 24hrs followed by the test agent and methyl-^{3}H thymidine for a further 48hrs. Incorporated label was measured initially according to the method of Morgan, (1987) but with subsequent modifications. Incorporated label was precipitated by the addition of trichloroacetic acid (TCA) and unincorporated label washed away. The precipitated material was then solubilized with concentrated formic acid, placed in scintillation fluid and counted using a liquid scintillation counter.

Preparation of culture plates and addition of cells and test agents

Initially 96-well plates, prepared as described in Section 2.6.1, were used for measuring methyl-^{3}H thymidine uptake. BREC were seeded at 4000 cells per well in 50μl DMEM containing 10% PDS into wells containing 100μl DMEM with 10% PDS and allowed to attach overnight. In some experiments cells were allowed to grow to confluence and were washed with 200μl HBSS per well. 200μl serum-free DMEM was then added to each well for 24hrs after which the test agent was added at the appropriate concentration in 200μl of fresh serum-free DMEM. Wells containing serum-free DMEM alone were used as controls. After a further 24hrs, 10μl of a 1:10 dilution of methyl-^{3}H thymidine (37 MBqml^{-1}) (Amersham) was added to each

well and the plates incubated at 37°C for a further 24hrs. In other experiments, cells were seeded at 4000 cells per well and after allowing the cells to attach for 24hrs, 10μl methyl-^{3}H thymidine solution was added as above along with the test agent or DMEM containing 10% PDS as a control and the cells incubated for 24hrs. To harvest the incorporated label, the medium was removed from the wells which were washed 3x with 200μl of PBSA. 200μl of 10% w/v TCA was added to each well for 5mins followed by 200μl of 5% w/v TCA for a further 5 minutes to precipitate out insoluble material and wash away unincorporated label. 200μl of methanol was added to each well to fix the precipitated material which was solubilized by the addition of 200μl of 25M formic acid. The acid digest was removed from each well and added to 1ml of liquid scintillation fluid (Optiphase Safe, LKB, Pharmacia) in scintillation vials (Beckman, High Wycombe, U.K.). The wells were washed with 200μl of PBSA and this wash added to the appropriate scintillation vial. The radioactivity in each vial was measured using a liquid scintillation counter (LS 1800, Beckman).

For a later series of experiments methyl-^{3}H thymidine incorporation was measured in BREC grown in 24-well plates in order to overcome the problems of poor precision seen when 96-well plates were used. 24-well tissue culture plates were prepared as described for 96-well plates except that 100μl of fibronectin (50 $\mu g ml^{-1}$) per well was used.

Optimized protocol for measuring cell proliferation by methyl-^{3}H thymidine incorporation

24-well culture plates were prepared as described above. 400μl DMEM containing 10% PDS was added to each well. BREC were then trypsinized, counted and diluted to 50,000 $cells ml^{-1}$. 100μl of this suspension was added to each well to give a final concentration of 5000 cells per well. After 24 hrs the medium was removed and the wells washed once with HBSS. 0.5ml of DMEM containing 0.1% PDS was added to each well and after a further 24 hour incubation the medium was removed, the cells washed once with HBSS and DMEM containing 0.1% PDS and

10 μlml^{-1} of the following mixture :- 60μl 10mM thymidine, 600μl methyl-^{3}H thymidine and 1340μl sterile H_2O, was added to each well. Test agents were added in an appropriate volume of DMEM containing 0.1% PDS and the plates incubated for 48hrs. The medium was then removed and the wells washed 3x with 0.5ml PBSA followed by the addition of 0.5ml 5% w/v TCA for 5mins. 0.5ml of 95% v/v ethanol was added to each well and removed before the addition of 0.5ml of 25M formic acid. The acid digest was placed in 3.5ml of liquid scintillation fluid and each well washed with 0.5ml PBSA which was added to the appropriate scintillation vial. The radioactivity in the vial was measured with a liquid scintillation counter.

2.6.3 Cell counting

Principle

Cell counting is generally regarded as the reference method for measurement of cell proliferation because of the direct relationship between cell number and the amount of cell division that has taken place.

Two methods were used to count cells. The first used the haemocytometer (Improved Neubauer, Weber Scientific, Teddington, U.K.) which is a glass slide with an H-shaped groove enclosing an upper and lower area. A grid is marked in the center of each area. When a cover slip is stuck to the slide so that interference patterns can be seen at the points of attachment, the cover slip is 0.1mm above the slide and the volume of liquid introduced beneath the coverslip by capillary action is defined by the squares of the grid. The central square of each grid, which is the largest area bounded by 3 parallel lines is 1mm^2 so the volume enclosed by these lines and the coverslip is 1mm^3 or 10^{-4} ml. Cells in the liquid can be counted under a phase contrast microscope as either total cells or as viable and non-viable cells after the addition of Trypan blue dye which is excluded from viable cells.

The second method used a Coulter counter (Coulter Electronics, Luton, U.K.). This instrument counts cells by

passing a fixed volume of cells through a small orifice in a glass probe. The glass probe is placed in an isotonic solution containing the cells being counted. It is also connected to a pump and a mercury manometer by a two way valve. When the manometer is connected to the pump, the mercury is drawn up to a starting position, when it is disconnected the mercury moves back down the tube and draws solution containing the cells through the orifice. The mercury in the other arm of the manometer moves up and passes two switch points, the first starts the count and the second stops it. The distance between the switches is calibrated so that a known volume of cell suspension passes through the orifice. A current is applied across the orifice and as the cells move through it they change the resistance to the current which generates a pulse that can be detected and quantified. The size of the pulse is proportional to the volume of the cell and can be viewed on a cathode ray oscilloscope. Another instrument used in conjunction with the Coulter Counter was a Channelyser (Coulter Electronics). This allowed the range of cell sizes being counted to be examined. Upper and lower threshold controls on the Coulter Counter could be adjusted to define a window of cell size. Only cells whose size falls within this window will be counted so the interference of small particles and cells of a different size can be eliminated. The amplification and the current setting can also be adjusted to the correct values for a particular cell size.

Preparation of cells

The critical requirement for cell counting using either a haemocytometer or a Coulter Counter was the production of a single cell suspension. Cells grown in flasks or multiwell plates could be counted although a minimum number of cells was required by the Coulter Counter for the count to be precise. This prevented the counting of cells grown in 96-well plates. If the counts were low when using the haemocytometer, precision could be increased by counting the cells in a greater number of grid squares.

Two methods were used for preparing a single cell suspension. The first was a standard trypsinization procedure (see Section 2.5.2) except that the initial centrifugation step was omitted. This prevented the loss of floating clumps of cells which were not pelleted by centrifugation. The cells were finally suspended in 1ml of HBSS. The second method was used primarily with multiwell plates where it was more difficult to remove all the cells from the substrate because the plates could not be hit as hard against the palm of the hand to dislodge the cells as could sealed flasks. For 6-well multiwell plates, the supernatant was removed and the cells washed once with 1.5ml of HBSS. A further 1.5ml of HBSS was added and the plates incubated at 37°C for 10mins. The HBSS was removed and 1ml 0.25% trypsin per well was added for 2mins at 37°C. The plate was then gently tapped to dislodge cells and these were removed and placed in a 30ml universal container containing 2ml of DMEM and 20% CS to inactivate the trypsin. 1ml of a $1mgml^{-1}$ solution of collagenase (Boehringer Mannheim) was added to each well. The collagenase removed the cells still attached to the bottom of the well and these were added to the cells from the same well. The cells were centrifuged at 325g for 5mins, the supernatant rapidly poured away and any supernatant adhering to the sides of the container wiped away with a tissue. 300μl of collagenase ($1mgml^{-1}$) was then added to each container and the cell pellet was resuspended by gentle agitation. A combination of shear forces in the liquid and the action of collagenase weakened the cell to cell attachments. 700μl of DMEM containing 20%CS was added to each container to neutralize the action of the collagenase and the cell clumps were broken up by repeated pipetting of the cells through a pipette tip using a 1ml variable pipette (Finnpipette, Labsystems, Basingstoke, U.K.).

For counting BRP it was not necessary to use collagenase as they did not produce large amounts of ECM. The trypsin was left on the cells for 30-35mins or until all the cells had detached. The cells were suspended in 1ml of DMEM + 20% calf serum and clumps broken by pipetting as described above.

Haemocytometer counting

100μl of cells suspended in HBSS were mixed with 100μl of 0.4% v/v trypan blue and the solution was allowed to run from a pipette tip, by capillary action under a coverslip attached to a haemocytometer slide. The cells in five 1mm squares were counted using a phase contrast microscope at a magnification of x100.

Coulter counting

The settings on the Coulter counter optimized for counting BREC were as follows:- Count volume - 500μl; Orifice size - 100μm; Lower threshold - 5; Upper threshold - infinity; Amplification - 8; Aperture current - 1. The counter was flushed with Isoton (Coulter Electronics) before use. An appropriate volume (100-500μl) of the cell suspension, which depended on the concentration of cells being counted, was added to 10ml of Isoton in a cuvette, and 500μl of the suspension counted at least three times.

2.7 Messenger RNA and Northern blotting

Principle

A complementary DNA (cDNA) probe for bFGF, isolated from a bovine pituitary cDNA library (Abraham et al., 1986a), was a gift of Dr. J.A.Abraham and Dr J.C.Fiddes, California Biotechnology Inc., California, U.S.A. The probe was a strand of DNA with a base sequence complementary to that of bFGF messenger RNA (mRNA) which allows it to hybridize to bFGF mRNA. When labelled with a marker, for example radioactive ^{32}Phosphorous (^{32}P), it can be used to detect mRNA transcripts for bFGF in extracts of cells and tissues. It had been ligated into a plasmid. Plasmids are circular pieces of double stranded DNA which can be inserted into bacteria and are replicated as the bacteria multiply. They also contain antibiotic resistance genes which allow a clone containing the plasmid to be isolated. This enables large quantities of

the plasmid containing the probe to be produced. Competent E.Coli were transformed by the plasmid, grown on agar plates containing the antibiotic penicillin and those clones that had been transformed by the plasmid were isolated. Bacteria from these colonies were grown in sufficient quantity for stocks to be produced and these were stored at -70°C. The remaining bacteria were lysed and the plasmid purified by density gradient centrifugation. The probe was then cut from the plasmid using restriction enzymes and separated from the plasmid DNA by agarose gel electrophoresis. The probe was isolated from the gel and stored at -20°C until used.

Total RNA was isolated from cultured cells by the guanidinium thiocyanate (GTC)/ caesium chloride method (Chirgwin et al., 1979). Known amounts of RNA were denatured using glyoxal and DMSO and the RNA species present separated according to their molecular size, by agarose gel electrophoresis. DNA restriction fragments of known size labelled with ^{32}P were also separated on the gel so that the size of the mRNA transcripts could be determined by comparison. The RNA was transferred to nylon membranes by capillary transfer (Northern blotting, Thomas, 1980). The cDNA probes were labelled with ^{32}P by random primer labelling and hybridized to the RNA on the filter. Probe bound non-specifically to the filter or to non-complementary RNA was washed off under conditions of increasing stringency, raised temperature and decreased salt concentration of the wash solution. Probe bound to the filter was visualised by autoradiography. The probe was removed from the filter by washing in a low salt buffer at high temperature. The filter was then reprobed with a ^{32}P labelled oligonucletide probe for actin. Actin mRNA is expressed at constitutively high levels in cells and these are unlikely to be altered by cell treatment. By comparing bFGF and actin signal strengths the effects of different loading and transfer efficiencies between RNA from different treatment groups could be eliminated.

2.7.1 Plasmid preparation

The cDNA probe for bFGF was a 1.4 Kb fragment ligated into the EcoR1 site of the plasmid pBR322. The resulting plasmid was called pJJ11-1 (Abraham et al., 1986a). The plasmid was used to transform the competent E. coli strain JM109 in order to produce sufficient stocks of plasmid for future probe preparation.

E. coli culture

Agar plates were prepared by warming 32g LB-Agar (Gibco) and 5g NaCl per litre of sterile distilled water until dissolved. The solution was autoclaved and 10ml of sterile 1M $MgSO_4$ was added. The cooling agar was poured into sterile petri dishes (Sterilin) and allowed to set. Agar plates were stored in sealed polythene bags at 4°C until used. E. coli were transferred to the plate by removing colonies of bacteria from a previous culture with a sterile loop and smearing them gently across the surface of the agar. The plates were incubated, upside down to prevent condensation falling on the growing colonies, at 37°C overnight. Plates containing colonies were sealed with sealing film and stored inverted at 4°C. Colonies were passaged every 2 months as described above.

Transformation with the plasmid

LB-broth was prepared by dissolving 10g powdered LB-medium (Gibco) in 500ml distilled H_2O. The solution was autoclaved and 5ml each of the following sterile solutions was added, 1M $MgCl_2$, 1M $MgSO_4$ and 2M glucose. E. coli colonies from a fresh overnight growth were scraped from the plate and placed in 20ml of LB-broth in a 50ml sterile container (Falcon, Marathon Labs., London, U.K.). These were placed at 37°C in a G25 shaking incubator (New Brunswick Scientific, Edison, U.S.A) for 5hrs. A sample was removed and the optical density at 600nm measured with a spectrophotometer (SP1800, Pye Unicam, Cambridge, U.K.). An optical density of about 0.3

indicated sufficient bacterial growth and the cells were centrifuged at 475g for 10min at room temperature. The supernatant was discarded and the cells resuspended in 2ml sterile 50mM $CaCl_2$. A further 18ml of 50mM $CaCl_2$ was added and the tube placed on ice for 30min. The tube was spun at 475g for 10mins at 4°C and the supernatant discarded. The cells were resuspended in 4ml ice cold 50mM $CaCl_2$ and left overnight at 4°C. This treatment makes the plasmid DNA stick to the bacterial membranes where it is more readily taken up. Two tubes were prepared, one with 1μl of solution containing 50ng of plasmid and 100μl of the treated E. coli suspension and the other with 100μl of treated E. coli suspension alone. Both tubes were placed on ice for 30mins and then heat shocked by placing in a water bath at 42°C for exactly 45 seconds during which time the plasmid enters the bacterium. They were then chilled on ice for 2mins and 900μl of LB-broth was added to each tube. The tubes were incubated at 37°C in a water bath for 1hr and centrifuged at 475g for 5min. The supernatant was decanted and the cells resuspended in 200μl of LB-broth. Each cell suspension was plated onto an agar plate prepared as described above but with the addition of 25μgml^{-1} ampicillin. The plates were incubated overnight at 37°C and examined for the presence of bacterial colonies. Only those bacteria transformed by the plasmid grew on the ampicillin containing plates, the untransformed cell plate acted as a negative control. The plate containing the transformed colonies could be stored sealed at 4°C for up to 3 months.

Extracting the plasmid

1.5 litres of LB-broth were prepared as described but 25μgml^{-1} ampicillin was added after the autoclaved solution had cooled. A scrape of the transformed colonies was placed into 20ml of LB-broth containing ampicillin and incubated for 6hrs at 37°C and 200rpm in a shaking incubator. This suspension was used to inoculate 1litre of LB-broth and this was incubated overnight at 37°C and 200rpm. The overnight growth was centrifuged at 500g in large capped flasks in a

MSE 4L centrifuge (Fisons, Crawley, U.K.) for 10mins. The supernatants were discarded and the rest of the procedure was carried out on ice. The bacterial pellet was resuspended in 8ml of 10% w/v sucrose in 50mM Tris/HCl pH 8.0 and the suspension transferred to an Oakridge tube (BDH). 1ml of lysozyme ($30mgml^{-1}$) was added and the tube left on ice for 10mins. 2ml of 0.5M EDTA pH 8.0 was added for 5min followed by 5ml of 1% v/v Triton-X-100 (Sigma). The solution was mixed and warmed to 37°C for 2min, at this time cell lysis occurred. The tube was centrifuged at 23,500g for 90min at 4°C. The supernatant was poured into a 50ml sterile tube and made up to 20ml with distilled H_2O. 19g of CsCl was added and mixed by inversion. 400μl of ethidium bromide ($10mgml^{-1}$) was also added and the solution again mixed by inversion. The tube was centrifuged at 925g for 5min at room temperature and the top protein layer was removed with a sterile swab. Two quick seal tubes (16 x 76mm, Beckman) were filled with the supernatant and heat sealed. These tubes were then spun at 158,000g overnight at 20°C in a Ti70 fixed angle rotor and L8-80M ultracentrifuge (both Beckman). After the overnight spin the rotor was decelerated without breaking to prevent disrupting the layered bands. The cellular material had resolved into several zones visualized with the ethidium bromide. At the top of the tube was protein, at the bottom a pellet of genomic DNA and in the middle two bands, the upper of RNA and the lower of plasmid DNA. The plasmid DNA band was removed by inserting a 21 guage needle into the top of the tube to admit air and another, connected to a syringe directly below the plasmid band which was sucked into the syringe. It was placed in a 13 x 51mm quick seal tube which was topped up with 0.95 gml^{-1} CsCl containing ethidium bromide and the tube was heat sealed. It was centrifuged at 218,500g in a VTi 80 vertical rotor (Beckman) overnight at 20°C. This spin produced a much wider separation between the plasmid DNA band and the RNA band than the previous spin and the plasmid DNA was removed as above. A solution was prepared containing 45ml isopropanol and 5ml TE (10mM Tris, 1mM EDTA pH 8.0). This was added 1:1 to the plasmid band and mixed by inversion. The ethidium bromide partitioned into the solvent

layer leaving the DNA in the aqueous CsCl layer beneath. All the solvent was removed and the process was repeated twice more. 3/4 inch dialysis tubing (Visking,BDH) was prepared by rinsing with distilled water and the plasmid solution added. It was dialysed overnight at 4°C against 4 litres TE followed by a further overnight dialysis at 4°C against 1:9 dilution of TE. The plasmid solution was removed and concentrated to half volume in a Savant vacuum centrifuge (Damon/IEC, Dunstable, U.K.). The concentration of plasmid in the solution was estimated by diluting 5μl to 400μl in distilled water and reading the optical density at both 260 and 280nm with a spectrophotometer. For a pure plasmid preparation the ratio of optical densities at 260 and 280nm should be 1.8-2.0 or greater and the concentration in $\mu g ml^{-1}$ is the optical density at 260nm x 50 x the dilution. The plasmid preparation was stored at -20°C.

Preparation of glycerol stocks

0.8ml of the bacterial suspension from the overnight growth in LB-broth containing ampicillin was mixed with 0.2ml of sterile glycerol and the stock stored at -70°C for use in subsequent plasmid preparations.

2.7.2 cDNA probe preparation

Plasmid digest

1μg of plasmid DNA containing the bFGF cDNA probe insert was mixed with 4 units (1μl) of the restriction enzyme Eco R1, 1μl of the matched buffer solution (H) (both Boehringer) and was made up to 10μl with sterile distilled H_2O in a sterile 1.5ml microfuge tube (Greiner Labortechnik). This was incubated for 2hrs in a water bath at 37°C. The restriction enzyme cut the plasmid at the Eco R1 sites where the bFGF probe had been inserted. The enzyme was inactivated at 68°C for 10mins and the tube placed on ice.

Pouring a minigel

A stock gel was prepared by mixing 1g Ultrapure, DNA grade, agarose (Biorad, Hemel Hempstead, U.K.) per 100ml of 1x TBE (50mM Tris, 50mM boric acid and 1.0mM EDTA) the agarose was dissolved by heating in a microwave oven (BDC, London, U.K.) for 4mins and 10μl ethidium bromide (10mgml^{-1}) was added per 100ml. The solution was poured into 50ml sterile tubes and allowed to set. The tubes were wrapped in foil to prevent deterioration of the ethidium bromide by light and the gel stored at room temperature. For use the gel was heated in a microwave for 3min and poured into a gel electrophoresis block containing an 8-slot comb. The open sides of the block were taped with autoclave tape. The gel was allowed to set level in the electrophoresis tank, the autoclave tape was removed and the gel covered with 500ml 1x TBE. The comb was removed and the samples prepared for loading.

Loading and running the gel

Samples were prepared by mixing either the plasmid digest, 1μg of uncut plasmid or 1μg of the molecular weight markers prepared as described in Section 2.7.3 with 1μl of bromophenol blue dye. This was a mixture of 10mg bromophenol blue, 10mg xylene cyanole, 1.2ml glycerol, 0.4ml 10x TBE and 2.4ml H_2O. The samples were loaded into seperate slots in the gel and this was run at 110 V for about 1hr during which time the dye front moved about half way up the gel. The power was disconnected and the gel block containing the gel removed. The migrated bands of DNA were examined by U.V. transillumination and photographed.

Isolating the DNA fragments

The two bands corresponding to the plasmid DNA and the probe DNA were distinguished according to their size, estimated against the molecular weight markers (see Fig. 2.2). The excised bFGF cDNA probe is 1.4kB and the remaining

plasmid 4.4kB.

DEAE cellulose membrane (NA-45, Anderman) was prepared by washing for 10min in 10mM EDTA pH 7.6, 5min in 0.5M NaOH and rapidly several times in sterile distilled water in order to increase its binding capacity. It was stored in sterile distilled water at 4°C. A piece of membrane was cut to size and inserted into an incision in the gel just above the cDNA probe band (Fig. 2.2).

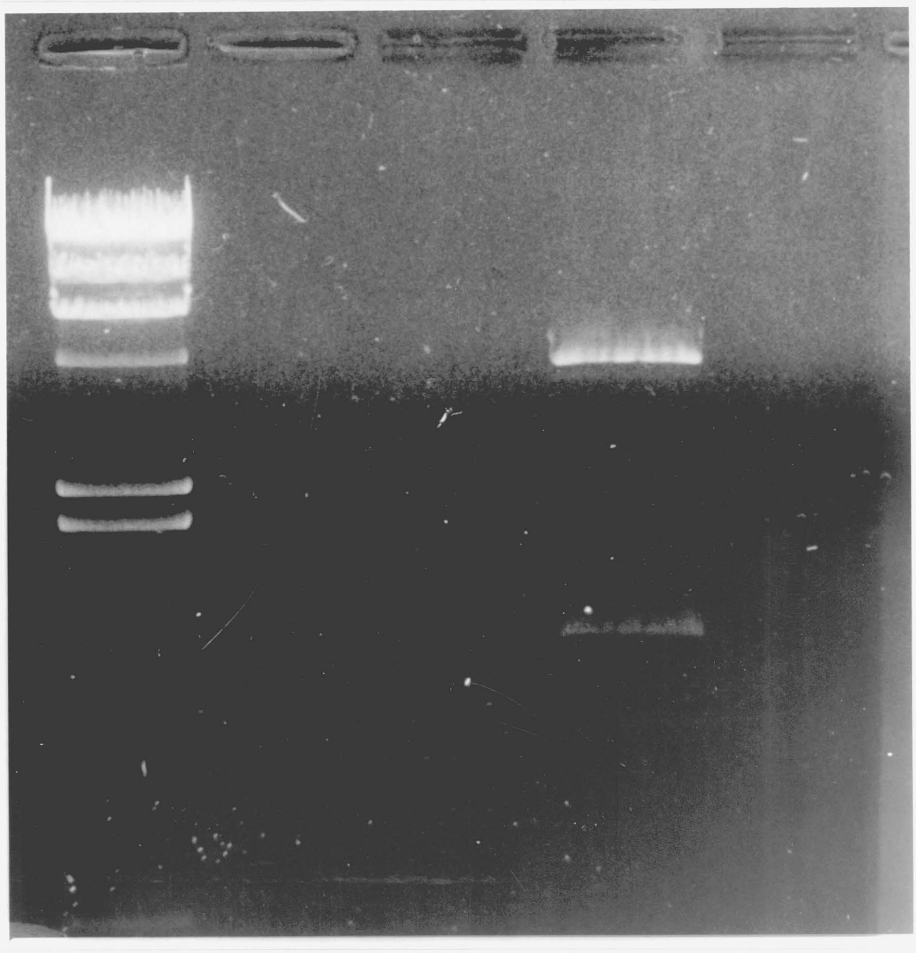


Fig 2.2 Agarose/ethidium bromide minigel viewed under U.V. light showing molecular weight markers in lane 1 and bFGF plasmid digest in lane 4

The gel block was then placed back into the electrophoresis tank and the power reconnected. The DNA band was allowed to move into the membrane as visualized with a hand held U.V. illuminator, the power disconnected and the membrane removed. Residual agarose was washed off the

membrane by shaking in a tube containing 0.15M NaCl, 0.1mM EDTA, 20mM Tris, pH 8.0 (NET) The membrane was placed in 150μl high salt NET (1M NaCl, 0.1mM EDTA, 20mM Tris, pH 8.0) and incubated in a water bath at 65°C for 45mins to elute off the DNA. The membrane was removed and discarded and 150μl H_2O, 2μl glycogen (20 $mgml^{-1}$, Boehringer Mannheim) and 750μl 100% v/v ethanol added to the tube. The tube was placed at -70°C overnight to precipitate the DNA and centrifuged at 15,600g in a microfuge (Damon/IEC) in a cold room for 30mins. The supernatant was removed and the surface of the DNA pellet washed with 1ml 70% v/v ethanol. The tube was centrifuged for 15min in the cold room, the supernatant poured off and the DNA dried in a vacuum centrifuge. The cDNA probe was resuspended in sterile distilled water and stored at -20°C until used.

2.7.3 Molecular weight markers

Molecular weight markers for running on Northern blots (Section 2.7.6) and alongside plasmid DNA (Section 2.7.2) were prepared by digesting bacteriophage lambda DNA (BRL, Paisley, U.K.) with the restriction enzyme HindIII. 25μg DNA in 100μl sterile distilled water was incubated at 37°C for 2 hours with 12μl matched buffer and 7.5μl (112U) HindIII (both U.S.B, Cleveland, Ohio, U.S.A) and the reaction stopped by heating at 68°C for 10mins.

2.7.4 Labelling procedures

Principle

Several methods were used for labelling depending upon the DNA being labelled. The requirements were the same in each case, to incorporate ^{32}P into DNA to a high specific activity. For the cDNA probe random primer labelling was used. The double stranded probe was denatured and incubated with a mixture of oligonucletide primers, dATP, dGTP, dTTP, $\alpha^{32}P$-dCTP and the Klenow fragment of DNA polymerase I. The primers hybridize to the denatured DNA and complementary

strands are synthesized from the 3'OH termini, catalysed by the Klenow enzyme and incorporating the ^{32}P labelled nucleotide. Unincorporated ^{32}P is then seperated from labelled probe using a DNA binding column (Elutip-d, Anderman).

Rat cytoplasmic B-actin oligonucleotide was labelled by 5' end labelling. The enzyme T4 polynucleotide kinase was used to catalyse the transfer of ^{32}P from $\gamma^{32}P$-dATP to the 5'OH terminal of the oligonucleotide. Purification was carried out with the DNA binding column.

The Hind III digested bacteriophage lambda molecular weight markers were also labelled by 5' end labelling using the enzyme T4 polynucleotide kinase. The transfer reaction described above could not be used because of the requirement for a free 5' OH group. On the DNA restriction fragments this is occupied by a terminal phosphate. However, in the presence of an excess of ATP and ADP, use can be made of T4 kinase's phosphatase activity to exchange this 5' phosphate for ^{32}P labelled gamma phosphate of ATP. Unincorporated label was again removed using a DNA binding column.

Random primer labelling

A random primed DNA labelling kit was used (Boehringer Mannheim). 25ng of cDNA probe were denatured by placing in a boiling water bath for 2mins and then rapidly chilling on ice. 1μl each of dATP, dGTP and dTTP (all 0.5mM) were added followed by 2μl of the reaction mixture containing the hexanucleotide primers. 5μl of an aqueous solution containing 1.85 MBq $\alpha^{32}P$-dCTP (110 TBq/mmol, Amersham) was added and the total volume made up to 19μl with sterile distilled H_2O. The reaction was started by the addition of 1μl of Klenow enzyme, the solution was carefully pipetted to mix and incubated at 37°C for 30mins. The reaction was stopped by heating the mixture to 65°C for 10mins.

Removal of unincorporated ^{32}P

Labelled DNA, prepared by any of the labelling procedures, was purified using Elutip-d DNA binding columns.

A column was pre-washed after cutting off the tip with a razorblade by passing 2.5ml of high salt buffer (1M NaCl, 20mM Tris, 1mM EDTA, pH 7.5) (HSB) through the column with a 10ml syringe. The column was primed with 5 ml low salt buffer (200mM NaCl, 20mM Tris, 1mM EDTA, pH 7.5) (LSB). 1ml of LSB was added to the labelling reaction mixture and this was pushed onto the column followed by a 1ml wash of the reaction vial. Unincorporated ^{32}P was then washed from the column with 7ml HSB and the DNA eluted into a sterile 1.5ml microfuge tube with 400µl HSB.

5′ end labelling

1µl rat cytoplasmic B-actin oligonucletide probe (20pM) (Clontech, Palo Alto, U.S.A) was mixed with 1µl 10x forward buffer (0.5M Tris pH 7.6, 100mM $MgCl_2$, 50mM dithiothreitol, 1mM spermidine, 1mM EDTA stored in aliquots at -20°C), 1µl $\gamma^{32}P$-dATP (110 TBq/mmol, Amersham) and 1µl T4 kinase (Gibco/BRL). The mixture was incubated at 37°C for 20mins and then 68°C for 10mins. Labelled DNA was purified as described above.

5′ end labelling - exchange reaction

2µg Hind III digested bacteriophage lambda DNA in 10µl sterile distilled H_2O was heated to 68°C for 10mins to break up concatamers and placed on ice. 2.5µl of 10x exchange buffer (50mM imidazole-HCl, pH 6.4, 12 mM $MgCl_2$, 15mM ß-mercaptoethanol, 0.3mM ADP and 0.5µM ATP) was added followed by 1.85MBq $\gamma^{32}P$ ATP (5µl), 1µl T4 kinase (10U) and 6.5µl sterile distilled H_2O. The mixture was incubated at 37°C for 20mins followed by 10mins at 68°C to inactivate the enzyme. Labelled DNA was purified as describe above.

Measurement of activity of labelled DNA

5µl of labelled DNA eluted from the DNA binding column was placed in 3.5ml liquid scintillation fluid and the radioactivity measured using a liquid scintillation counter.

2.7.5 RNA preparation

Principle

The first requirement of any RNA isolation procedure is the elimination of ribonuclease (RNAase) activity. RNAases are present in all cells and tissues, in the laboratory environment, on hands, glasswear and in sterile distilled water. Gloves were worn at all times, water was rendered RNAase free by treatment with diethylpyrocarbonate (DEPC) followed by autoclaving to inactivate the DEPC (DEPC-water) and glassware was rinsed with water containing DEPC followed by autoclaving. Chemicals and disposable plasticwear were purchased RNAase free. Cellular RNAases were denatured by lysing cells in 4M GTC containing detergent and mercaptoethanol. RNA was isolated from DNA and protein by differential sedimentation on a CsCl gradient in an ultracentrifuge. It was further purified by ethanol precipitation and was quantified and its purity determined spectrophotometrically.

Cell solubilization

Astrocytoma cells or bovine retinal endothelial cells cultured as described in Section 2.5, were trypsinized and the cell pellet dissolved in guanidinium thiocyanate solution (GTC, 50g guanidinium thiocyanate, 0.5g sodium lauryl sarcosine, 2.5ml 1M sodium citrate pH 7.0, 0.7ml ß-mercaptoethanol and 0.33ml antifoam A (Sigma). The mixture was made up to 100ml with DEPC-water, the pH adjusted to 7.0 and the solution filtered through an 0.45μ filter. A minimum of 6 x 10^6 cells were used per preparation solubilized in 7.5ml GTC. The GTC cell extract was stored at -70°C before use.

Density gradient centrifugation

5.7M CsCl was prepared by bringing 95.9g CsCl and 2.5ml 1M NaAcetate pH 5.2 to 100ml with DEPC-water and autoclaving

the resulting solution. 4ml of this solution was placed in the bottom of a 16 x 76mm quick seal centrifuge tube. The cell/GTC mixture was thawed and the viscosity reduced by passing the solution several times through a 21 gauge needle to shear the DNA. This allows the RNA to more readily pass through the GTC during centrifugation. The mixture was layered on the CsCl and when the tube was full it was sealed. Tubes were centrifuged at 70,200g in a Ti70 fixed angle rotor for 18hrs at 20°C with zero break setting. The RNA formed a clear pellet on the side close to the bottom of the tube.

RNA purification

The GTC and CsCl layers were removed with a syringe and needle, the bottom of the tube cut off with a razorblade. The tube was inverted and allowed to drain. The pellet was rinsed once with 95% v/v ethanol and the tube placed on ice. The pellet was resuspended in 100µl 0.1% w/v sodium dodecyl sulphate (SDS), prepared with DEPC-water and placed in a sterile 1.5ml microfuge tube. The centrifuge tube was rinsed with a further 100µl 0.1% w/v SDS and this was combined with the resuspended RNA. 66µl of 1M NaAcetate pH 5.2 was added and the tube vortexed. 2.7 volumes (718µl) of ice cold 100% v/v ethanol was added and the RNA precipitated either overnight at -20°C or on dry ice for 30mins. The tubes were centrifuged in a microfuge for 30mins at 4°C and the supernatant poured off. 1ml of 70% v/v ice cold ethanol was placed on the sample to wash off any remaining salts and the tube centrifuged at 15,600g for 15mins at 4°C. The supernatant was poured off and the RNA dried down in a vacuum centrifuge. The pellet was resuspended in 100µl of DEPC-water and the concentration and purity of the RNA assessed spectrophotometrically.

2.7.6 Electrophoresis and Northern blotting

Principle

A 1.2% w/v agarose gel was used which efficiently

separates denatured mRNA molecules with sizes between 0.4 and 6Kb. Purified RNA samples were denatured with glyoxyl/DMSO and loaded onto the gel alongside denatured DNA molecular weight markers prepared as described in Section 2.7.3. After electrophoresis the RNA was transferred to nylon membranes by capillary blotting.

RNA denaturation

Deionized glyoxal was prepared by stirring 20ml 40% w/v glyoxal (BDH) with 20g AG501-X8 ion-exchange resin (BioRad) at room temperature for 30mins and repeating the procedure with 4g of fresh ion-exchange resin until the pH was between 5.5 and 6.0. The solution was filtered through a pasteur pipette containing glass wool. Deionized glyoxal was stored in aliquots at -20°C and the pH checked before use to ensure that it was between 5.5 and 6.0. A solution containing 20μg RNA in a microfuge tube was dried in a vacuum centrifuge and redissolved in 3μl DEPC-water. 3μl deionized glyoxal was added followed by 7.5μl DMSO and 1.5μl 0.1M phosphate buffer pH 6.5 and the solution incubated at 50°C for 1hr and chilled on ice. Glyoxylated samples could be stored overnight at -20°C before use.

Agarose gel electrophoresis

The horizontal electrophoresis tank, block and comb (H4, Gibco/BRL) were rinsed with DEPC-water before use. 4.8g agarose was dissolved in 400ml 10mM phosphate buffer pH 6.8 using a microwave oven. The gel was allowed to cool and was poured into the taped gel block containing the comb and allowed to set. 2 litres 10mM phosphate buffer were added to the tank and the comb was removed. The glyoxylated RNA samples and the molecular weight markers (15μl) were each mixed with 3μl bromophenol blue dye (10mg bromophenol blue, 10mg xylene cyanole, 40μl 1M phosphate buffer pH 6.8, 1.2ml glycerol and 2.8ml DEPC-water) and loaded into the gel wells. The power was switched on (200V) and the dye front allowed to run a short way into the gel before switching on a

peristaltic pump (Flowgen, Sittingbourne, U.K.) which circulated the buffer through the apparatus and prevented the build up of pH gradients in the tank. Electrophoresis was carried out for 4-5 hours, the power switched off and the gel cut out of the block with a razor-blade. The bottom left hand corner of the gel was cut off in order to orientate the gel. A piece of nylon membrane (Nytran, Anderman) was cut to the same size as the gel and soaked in 10x SSC buffer (1.5M NaCl, 0.15M trisodium citrate) Two wicks were cut to the correct size from Whatman No.1 filter paper and laid over a clean glass plate which was placed over a metal tray containing 10x SSC buffer so that the wicks were hanging in the buffer. The gel was inverted onto the wicks and the nylon membrane placed on top of the gel. Two pieces of Whatman No.1 filter paper cut to the same size as the gel were placed on top of the nylon and 5-6cm of paper towels placed on top. Sealingfilm was placed around the gel to prevent buffer movement other than through the gel and a heavy weight was placed on top of the paper towels. Blotting was carried out for 18hrs at room temperature. The nylon membrane was baked at 80°C for 2hrs which partially removed the glyoxal and fixed the RNA to the membrane.

2.7.7 Hybridization of filter bound RNA to the cDNA probe

Principle

Hybridization involves the random collision of complementary sequences. Denatured single stranded RNA bound to the filter was hybridized to a ^{32}P labelled, cDNA probe. The double stranded cDNA was denatured by heating, so the reaction was a balance between reassociation of the probe and hybridization to the RNA. Factors governing the hybridization reaction are the temperature, the ionic strength of the hybridization solution and the presence of dextran sulphate which absorbs water and increases the concentration of the probe. The length of the probe, its guanine and cytosine base content and the percentage sequence mismatch between RNA and probe are also important. From these a formula can be derived

for the melting temperature, Tm, defined as the temperature at which the strands are half dissociated or denatured.

$$Tm = 69.3 + 16.6(\log_{10} M) + 0.41(\%G+C) - 500/L$$

where M is the molarity of the salt, %G+C is the percentage guanine and cytosine bases and L is the length of the duplex in base pairs. Every 1% mismatch reduces the Tm by about 1°C. In the hybridization buffer described below, bFGF cDNA has a Tm of 88°C, assuming an average probe length of >200 b.p. and a G+C content of 50%. Hybridization was carried out at 28°C below the melting temperature. Stringency washing was then carried out at 5°C below the hybridization temperature but with decreasing salt concentrations thereby lowering the Tm. This process removes mismatched hybrids. Blots were stripped by raising the temperature above the Tm and could then be reprobed.

Pre-hybridization

The nylon filter blot placed in a polythene bag the sides of which were sealed with a bag sealer (Pifco, BDH). Hybridization buffer (1M NaCl containing 1% w/v SDS and 10% w/v dextran sulphate), (1ml per cm^2 of the filter) was introduced through a hole in one corner of the bag with a needle and syringe. All air was excluded from the bag and the bag was resealed. It was incubated, submerged in a shaking water bath for at least 2hrs at 60°C.

Hybridization

The bFGF cDNA probe, labelled as described in Section 2.7.4, was denatured by heating to 100°C for 3mins and was then mixed with 2 volumes of hybridization buffer. It was added to the bag (10^5- 10^6 $cpmml^{-1}$) using a needle and syringe and the bag was resealed. The bag was checked for leaks and placed submerged in a metal tray in a water bath and incubated at 60°C for 18hrs. Rehybridization of the filter with the rat cytoplasmic ß-actin oligonucletide probe was

carried out by the same method after stripping the blot, see below.

Stringency washing

The filter was washed under conditions of increasing stringency (decreasing salt concentration) using dilutions of 20x SSC (3M NaCl, 0.3M trisodium citrate). It was first rinsed in 6x SSC and then washed in 6x SSC containing 0.1% w/v SDS at 55°C for 20mins and this was repeated. The concentration of SSC was then reduced to 2x and the filters washed twice under the same conditions as above. After these washes the filters were placed damp in a sealed polythene bag and the activity of the bound probe measured with a scintillation counter (Autograph, Saxon Micro, Newmarket, U.K.). If the non-specific signal was still too strong the filter was washed in 1x SSC containing 0.1% w/v SDS for 20mins at 55°C. The filter was rinsed in 6x SSC for 20mins at room temperature and then sealed in a polythene bag whilst still damp. The filter was not allowed to dry out if stripping and reprobing were to be carried out because this increased the background.

2.7.8 Detection of filter bound probe

Principle

Detection of the bound probe was carried out by autoradiography. Although equal amounts of RNA from cells in different treatment groups were loaded onto the gel it is still possible that different quantities were present on the filter due to different purities of the RNA preparation and to different transfer efficiencies during capillary blotting. The filter was therefore stripped and rehybridized with a probe for a high abundance transcript whose quantity did not alter with the treatment. The signal strength of the probe hybridized to this transcript was compared to the signal strength for the bFGF mRNA transcripts.

Detection of hybridized probe

The detection of the mRNA transcripts which hybridize to the probe was carried out by autoradiography. The hybridized filter was exposed to X-ray film (Kodak XAR5, Sigma) in a light proof cassette containing an intensifying screen (Sigma). The length of exposure depended upon the signal strength of the hybrids and exposure was carried out at -70°C because the fluorescence reflected off the intensifying screen is prolonged at low temperatures. The exposed film was developed with a Fujii RGII X-ray film processor (Fujii, Tokyo, Japan).

Stripping the filter

Filters were placed in a metal tray containing 500ml 10mM Tris pH 8.0 and 1mM EDTA and heated to 80°C for 10mins.

2.9 Statistics used

Unless otherwise indicated in the text, data means were compared using Student t-tests and multiple means by one way analysis of variance (ANOVA). The results were non-significant ($p>0.05$).

Chapter 3. DEVELOPMENT, CHARACTERISTICS AND PERFORMANCE OF THE IMMUNOASSAYS

3.1 Introduction

The aims of the thesis required a sensitive, specific and robust method for the measurement of fibroblast growth factor in clinical samples, retinal cell extracts and culture medium conditioned by retinal endothelial cells. Radioimmunoassay was chosen for this purpose because of its sensitivity and the facilities and materials available. When the project started, purified fibroblast growth factor was just becoming commercially available, however the cost of obtaining sufficient material for antibody production was prohibitive. We decided to work with basic FGF in preference to aFGF for two reasons. First, the amino-terminal amino acid sequence for bFGF was known and an RIA using antibodies raised against an N-terminal peptide sequence of bFGF had been reported (Baird et al., 1985c). Secondly, purification of basic and aFGF from bovine retina had shown the presence of equal amounts of these factors (Baird et al., 1985a). However, bFGF was reported as the more potent mitogen (Gospodarowicz et al., 1986b) and therefore constituted the majority of the fibroblast growth factor activity in this tissue. Both of the RIAs developed by us used antibodies raised against N-terminal sequences of bFGF although with different epitope specificities. The assays were characterized to assess their specificity and were optimized to maximise sensitivity and increase robustness.

The assays were used to measure bFGF in serum and vitreous samples from patients with and without proliferative diabetic retinopathy. The measurement of bFGF in bovine retinal extracts and the elution profile of these extracts on heparin-Sepharose was also carried out. Although similar work has previously been reported (D'Amore & Klagsbrun, 1984; Courty et al., 1985) these authors had used bioassays to measure growth factor activity eluted from the column rather than RIA. Information on the specificity of the RIA was obtained and this was extended by the use of immunoblotting.

Enzyme-linked immunoabsorbant assays were developed to improve both the specificity and the sensitivity of bFGF measurements particularly for with use with human vitreous samples.

3.2 RIA for bFGF using antibodies raised against (Tyr^{10})FGF(1-10)

3.2.1 Conjugation of (Tyr^{10})FGF(1-10) to BSA

The results of the conjugation reaction which provided material for the immunizations is shown in Table 3.1. ^{125}I-neurotensin was included in the same reaction mixture as (Tyr^{10})FGF(1-10) and BSA. This enabled the success of conjugation to be estimated. Free ^{125}I-neurotensin was separated from ^{125}I-neurotensin conjugated to BSA by activated charcoal adsorption.

Fraction	Sham reaction mixture (cpl0sec.)			Conjugation reaction mixture (cpl0sec.)		
	Total	NSB	Specific	Total	NSB	Specific
Pellet	1962	117	1845	1362	71	1291
Supernatant	66	48	18	946	121	825
%age Conjugation	$\frac{18}{1845 + 18} = 1\%$			$\frac{825}{1291 + 825} = 39\%$		

Table 3.1 An estimation of the success of peptide conjugation to BSA

3.2.2 Iodination of (Tyr^{10})FGF(1-10)

A typical HPLC profile for the separation of iodinated (Tyr^{10})FGF(1-10) from unincorporated label is shown in Fig.

3.1 The first peak is ^{125}I and the second peak iodinated peptide.

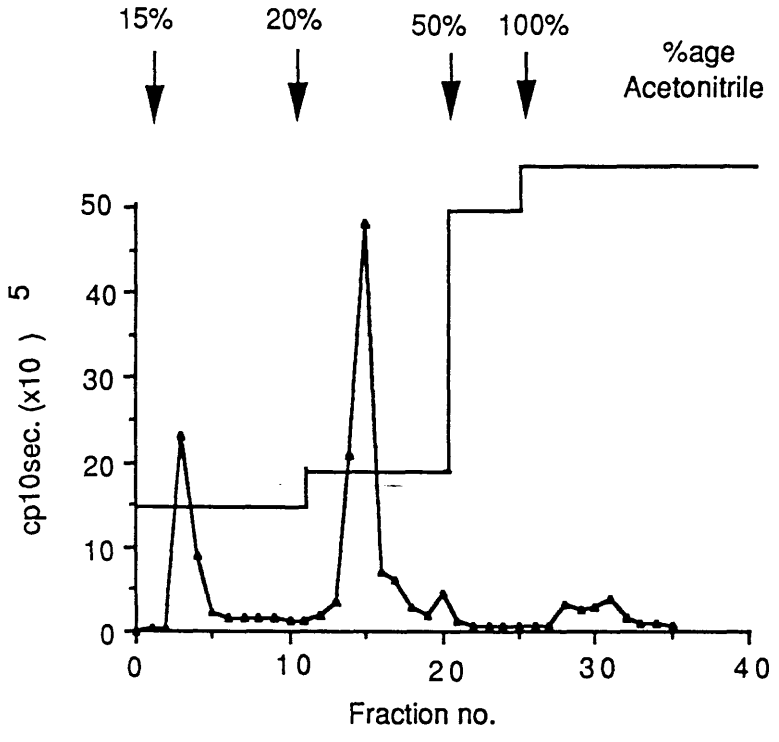


Fig. 3.1 HPLC purification of iodinated (Tyr^{10})FGF(1-10)

Using this data the specific activity of the labelled fraction was estimated as follows:-

$$\frac{\frac{\text{cp10sec.labelled fraction}}{\text{cp10sec. total}} \times \text{Total activity added (Bq)}}{\text{Total amount of peptide iodinated (fmol)}}$$

$$= \frac{\frac{92,196}{154,734} \times 37,000,000}{5,000,000} = 4.4 \text{ Bq/fmol}$$

3.2.3 (Tyr^{10})FGF(1-10) antibody titration

The results of the titration of antibody levels in serum from 3 rabbits bled at the end of the immunization schedule

is shown in Fig. 3.2. Using the antiserum showing the highest titre (rabbit 4), at a dilution of 1:1000, a standard curve was produced shown in Fig 3.3. Ethanol precipitation of the bound fraction was used for this standard curve.

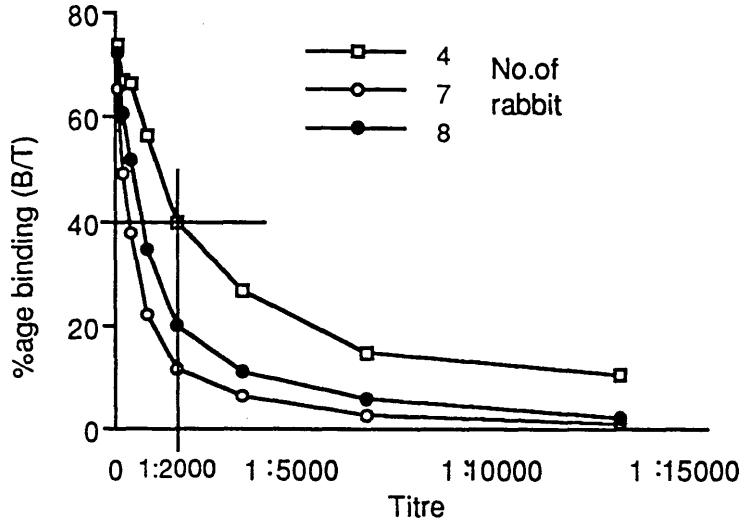


Fig. 3.2 Fraction bound against antibody dilution for immune sera from 3 rabbits

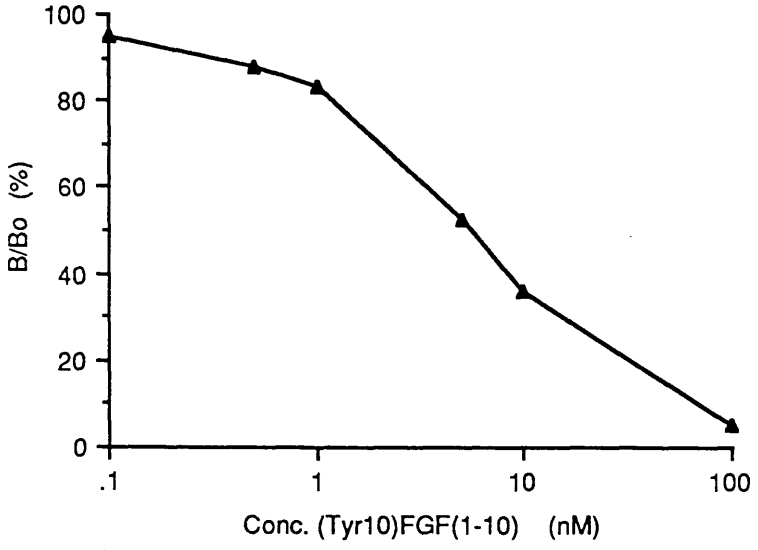


Fig. 3.3 Radioimmunoassay standard curve for (Tyr^{10})FGF(1-10)

In order to produce a more specific precipitation of the bound fraction a second antibody and non-immune serum titration series was carried out. The results, shown in Table 3.2 suggested appropriate dilutions of 1:20 for NIRS and 1:50 for GARS and these were used for the succeeding assays.

GARS NIRS	1:30	1:35	1:40	1:45	1:50	1:55	1:60
1:10	47.7	47.5	48.4	48.7	48.4	40.4	9.3
1:20	45.7	44.6	45.9	46.5	47.9	47.1	48.3
1:30	43.3	44.9	46.1	-	-	-	-
1:40	42.1	42.7	45.0	-	-	-	-

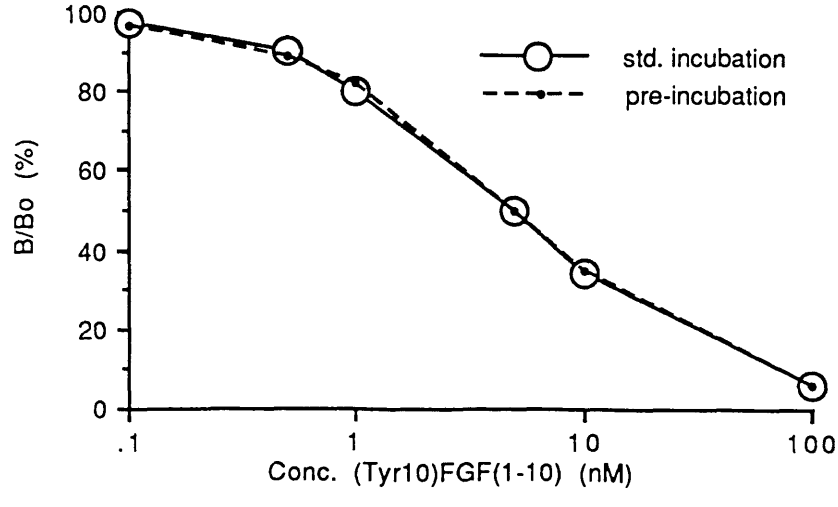
Table 3.2 Latin square antibody and non-immune serum titrations for (Tyr^{10})FGF(1-10) assay

3.2.4 Assay optimization

Optimization of the assay was carried out and the effects of varying some of the assay parameters is shown in Fig. 3.4.

Preincubation of sample and standard with antibody prior to the addition of label had no effect on the standard curve. Decreasing the amount of label added had only a small effect on apparent antibody titre and a label concentration of 15,000 cpm/100μl was chosen for the assay. Incubation time did not have a dramatic effect except when the 2nd antibody incubation was reduced to 2 hours. For convenience therefore an overnight 1st antibody incubation and a 4 hour 2nd antibody incubation were chosen. Assay sensitivity estimated as 3x standard deviations from the mean of B_0 was 115pM.

Fig. 3.4(a)



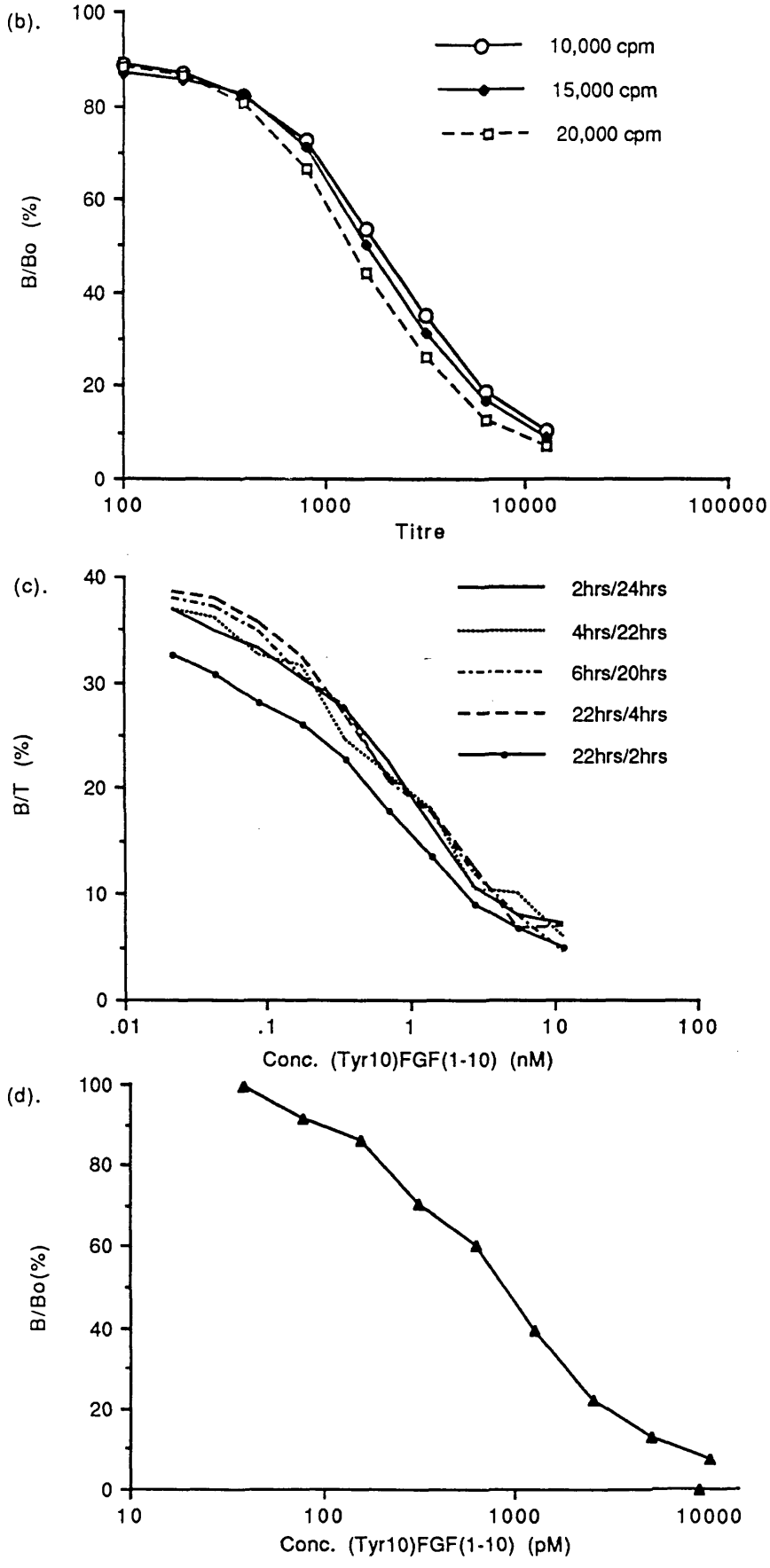


Fig. 3.4 The effect on the (Tyr^{10})FGF(1-10) assay of (a) preincubation, (b) changing the amount of added label and (c) altering the 1st and 2nd antibody incubation times is shown along with an estimate of assay sensitivity, (d)

3.2.5 Measurement of bFGF in serum and vitreous samples

Initial studies using the RIA produced values of 3.07 ± 0.42 nM (x±SEM; n=6; range 2.06-4.33) for serum from diabetic patients compared to 2.65 ± 0.15 nM (n=6; range 2.12-2.99) in serum from non-diabetic controls. Human vitreous samples showed bFGF immunoreactivity levels of 0.36 ± 0.11 nM (n=11; range 0.02-1.15). The level of bFGF immunoreactivity in the vitreous appeared to be related to the degree of contamination of the sample with blood. Rat serum showed a concentration of 2.50 ± 0.27 nM (n=4) and the values for rat vitreous and rat retina were 4.6 pmol/gm wet weight and 10.4 pmol/gm wet weight respectively.

When these preliminary results were being obtained, it was reported (Gauthier et al., 1987) that there was an aminopeptidase present in serum which cleaved two amino acids from the amino terminal of (Tyr^{10})FGF(1-10) producing a non-immunoreactive peptide. If the labelled peptide was similarly cleaved in the assay tubes containing serum or tissue extracts during the RIA incubation, less label would bind to the antibody and this would be mistaken for bFGF immunoreactivity in the samples. Enzyme activity could therefore be responsible for the apparent bFGF levels in serum and tissue samples. To test this, $^{125}I(Tyr^{10})$FGF(1-10) was added to samples of rat serum and vitreous. Its binding to the antibody at time T = 0 and 2 hrs is shown in Fig. 3.3.

Sample	% specific binding at time (hours)	
	0	2
Serum	13.3	1.6
Vitreous	2.9	0.3
Retina	22.4	0.0

Table 3.3 Assessment of $^{125}I(Tyr^{10})$FGF(1-10) degradation in rat serum, retina and vitreous

These results showed that aminopeptidase activity was present in these samples. The effect of a range of protease inhibitors on the assay standard curve is shown in Fig 3.5 and on the apparent bFGF immunoreactivity in plasma samples in Table 3.4.

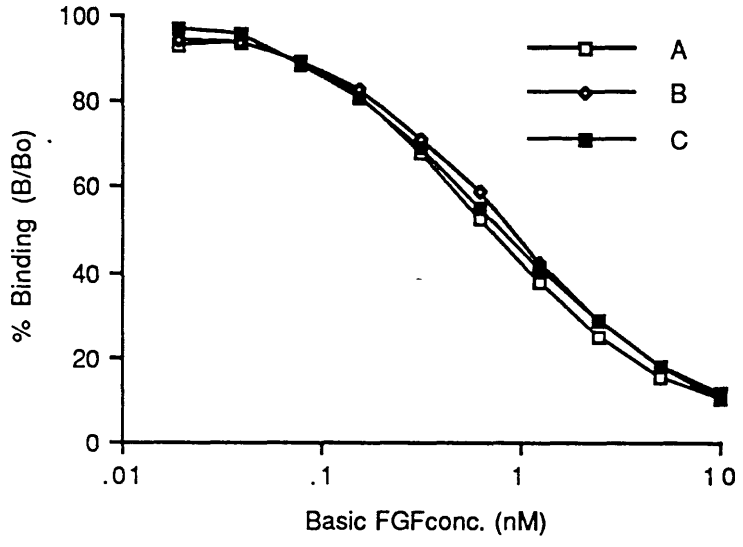


Fig. 3.5 The effect of protease inhibitors on the standard curve. Standards were prepared in A - Assay buffer, B - Assay buffer containing lima bean trypsin inhibitor (10μg/ml), bacitracin (200μg/ml), phenylmethylsulphonhylflouride (100μM) and trasylol (200 I.U./ml) and C - Assay buffer containing EDTA (4.1mM)

It was evident that a combination of bacitracin (0.8mgml^{-1}) and EDTA (4.1mM) was effective in inhibiting the aminopeptidase although heating to 75°C was not. A timed series followed the degradation of the peptide by the aminopeptidase in plasma samples in the presence of EDTA and an increasing concentration of bacitracin (Fig.3.6). This showed that bacitracin does inhibit the enzyme but that by 28 hrs. all ^{125}I(Tyr^{10})FGF(1-10) immunoreactivity has been lost indicating that the assay could not be used for the measurement of bFGF in these samples even in the presence of

protease inhibitors.

Sample	Apparent bFGF concentration(nM)
Plasma	>10
+ EDTA (4.1 mM)	7.9
+ Trasylol	>10
+ Lima bean trypsin inhibitor(10μg/ml) + EDTA (4.1 mM)	7.1
+ Bacitracin (0.8 mg/ml) + EDTA (4.1 mM)	0.2
+ PMSF (80μg/ml) + EDTA (4.1 mM)	7.1
Heating to 75°C	8.9

Table 3.4 The effect of protease inhibitors and heating on the apparent bFGF concentration of plasma samples

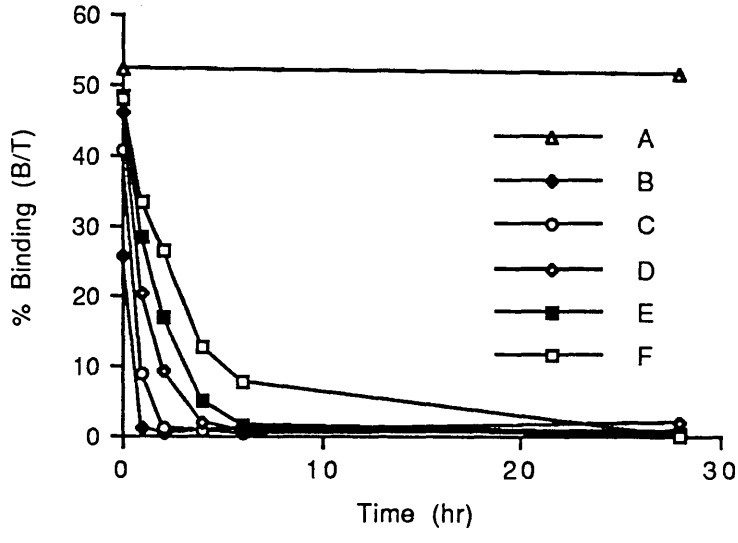


Fig. 3.6 Inhibition of enzyme activity responsible for the degradation of $^{125}I(Tyr^{10})FGF(1-10)$. (A = Assay buffer, B = heparin plasma, C = EDTA plasma + bacitracin (0.8 mg/ml), D = EDTA plasma + bacitracin (1.6 mg/ml), E = EDTA plasma + bacitracin (4mg/ml) and F = EDTA plasma + bacitracin (8mg/ml)

3.3 RIA for bFGF using an antibody raised against the amino-terminal 1-24 amino acids of bFGF(1-146)

3.3.1 Iodination of recombinant bFGF

A typical elution profile obtained during the purification of ^{125}IrbFGF on a heparin-Sepharose column is shown in Fig. 3.7.

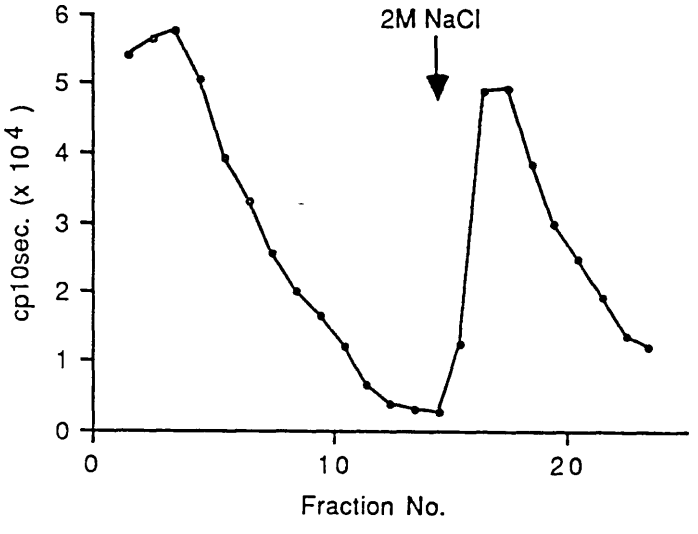


Fig. 3.7 Elution of ^{125}IrbFGF from a heparin-Sepharose column

The specific activity of the label was calculated from this profile and a measure of the counts remaining bound to the column. The specific activity for this and other preparations of label was 117 ± 14 Bq/fmol^{-1} (mean ± s.d.; n=3). The specific activity of pure ^{125}I is 81 Bqfmol^{-1} so this indicates that on average slightly more than one tyrosine per bFGF molecule was iodinated.

A lactoperoxidase method of iodination was used before changing to the chloramine-T method (see Section 2.1.3). The former is reported to produce less damage to the iodinated protein because milder reaction conditions are used. No differences were noted however between label iodinated by either method. HPLC purification was used in order to try to obtain label of a higher specific activity. It was found however that the 40% v/v acetonitrile and 0.1% v/v TFA in the

eluant damaged the iodinated rbFGF.

When ^{125}IrbFGF became commercially available a comparison was made between this and rbFGF iodinated by the chloramine-T procedure. Standard curves produced using these labels are shown in Fig. 3.8. The commercial ^{125}IrbFGF produced a standard curve shifted to the right with a consequent decrease in assay sensitivity. This could be due to its low specific activity of 52 Bqfmol^{-1}which the manufacturers state as being necessary to maintain stability. We routinely repurified our iodinated product so stability was not a problem and labels with a higher specific activity could be used.

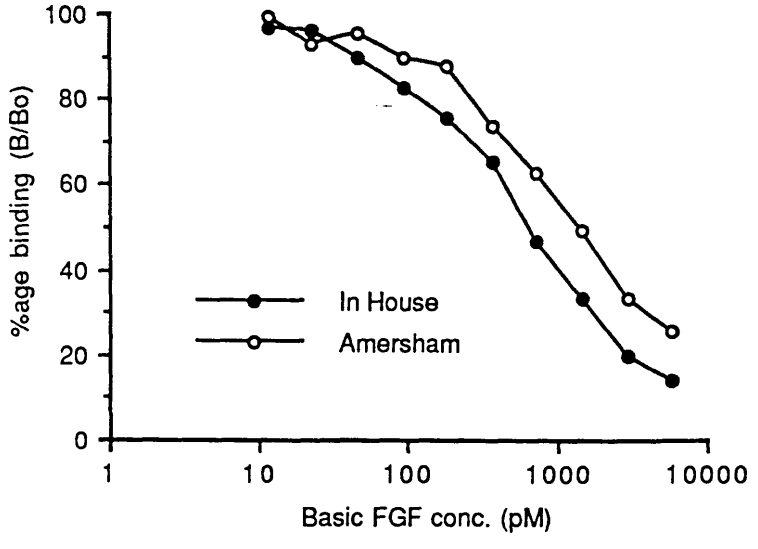


Fig. 3.8 Standard curves for rbFGF produced with commercial or in-house label

3.3.2. Optimization of the bFGF RIA

A non-immune serum and second antibody titration was carried out and showed that the optimum final dilutions were 1:200 for the non-immune rabbit serum and 1:500 for the goat anti-rabbit serum. A range of primary antibody dilutions, around that suggested by Dr. Baird, were tried and the results shown in Fig.3.9 along with the effect of varying the amount of label added, the incubation times for 1st and 2nd antibody, the pH of the buffer solution. The results of including a pre-incubation of 5hrs. prior to the addition of label and the standard curve used for estimating assay

sensitivity are also shown.

Minor effects only were seen with variations in the antibody concentration, label concentration and buffer pH. The values chosen for the optimized assay were a primary antibody dilution of 1:20,000, 10,000cpm per 100μl added label and a buffer pH of 7.2. The percentage binding was only markedly reduced when the primary antibody incubation time was reduced to 3 hours so the incubation times chosen for convenience were overnight for the 1st antibody and 4 hours for the 2nd antibody. The addition of a pre-incubation shifted the standard curve to the left however the effect at the lower standard concentrations was negligible and the sensitivity of the assay was not increased. An estimate of the assay sensitivity as 3 x the standard deviation from B_0 was 18 pM.

3.3.3 Assay characteristics

The cross reactivity of aFGF and IGF-1 in the RIA is shown in Fig. 3.10. It is clear that significant cross reactivity in the assay occurs only with aFGF at concentrations beginning at 2nM. Human growth hormone and two peptides showing some sequence homology with the epitope of bFGF (1-24) recognized by the antibody, ACTH and CRF, showed no cross reactivity in the assay.

Fig. 3.9 (a)

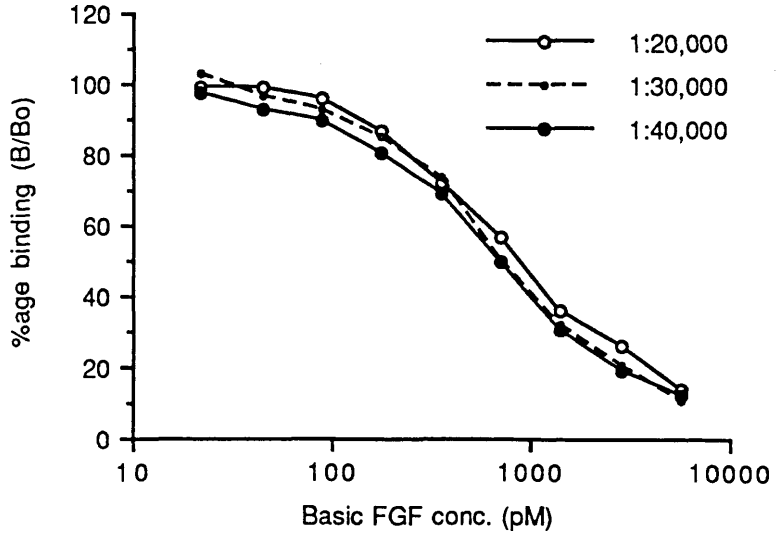
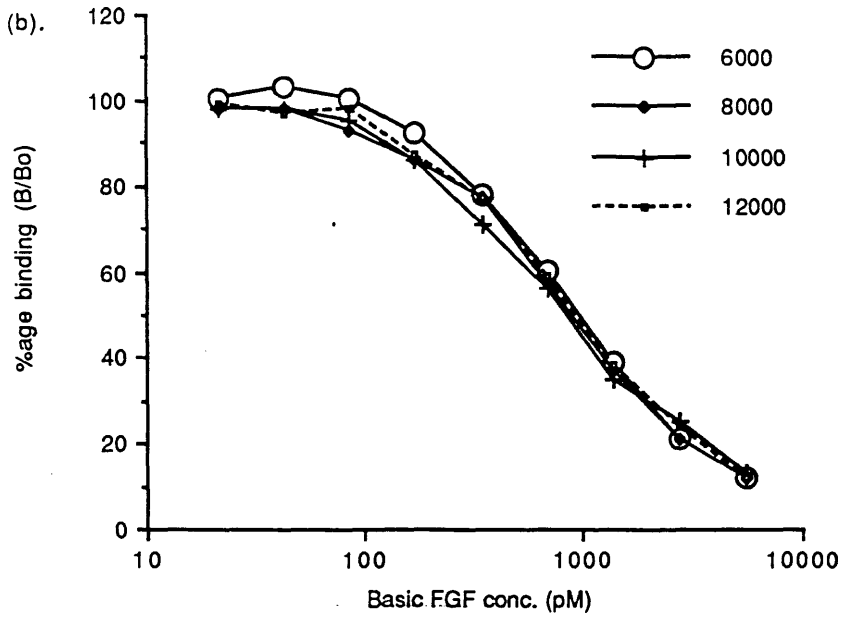
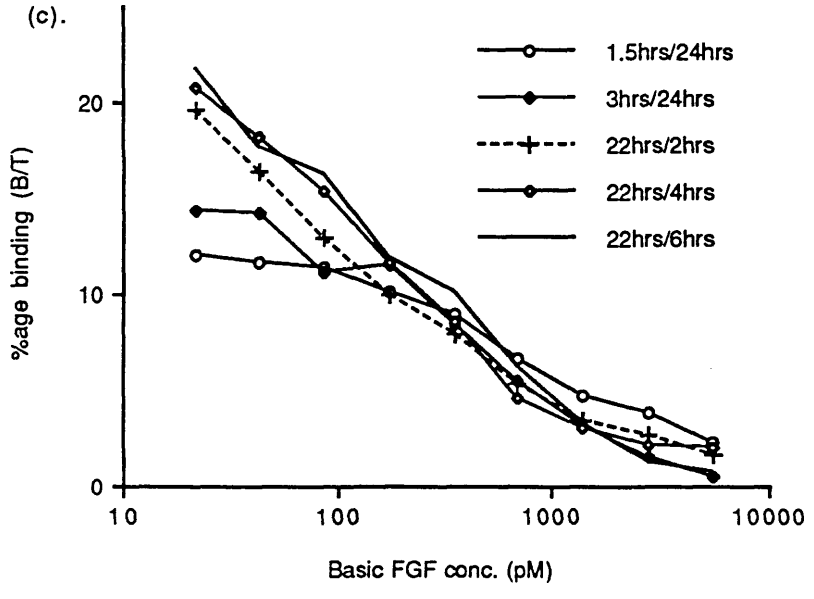
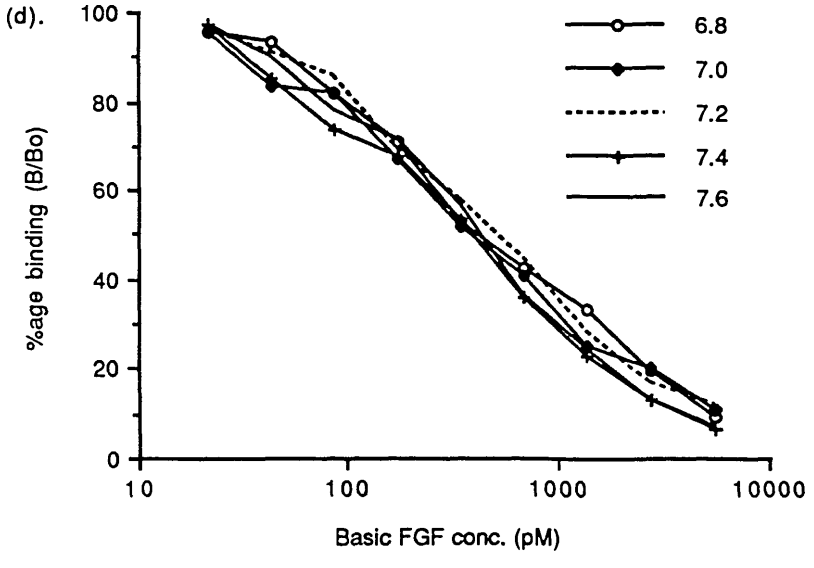


Fig. 3.9 (b-d)


(b).
%age binding (B/Bo)
120
100
80
60
40
20
0
10
100
1000
10000
Basic FGF conc. (pM)
6000
8000
10000
12000


(c).
%age binding (B/T)
20
10
0
10
100
1000
10000
Basic FGF conc. (pM)
1.5hrs/24hrs
3hrs/24hrs
22hrs/2hrs
22hrs/4hrs
22hrs/6hrs


(d).
%age binding (B/Bo)
100
80
60
40
20
0
10
100
1000
10000
Basic FGF conc. (pM)
6.8
7.0
7.2
7.4
7.6

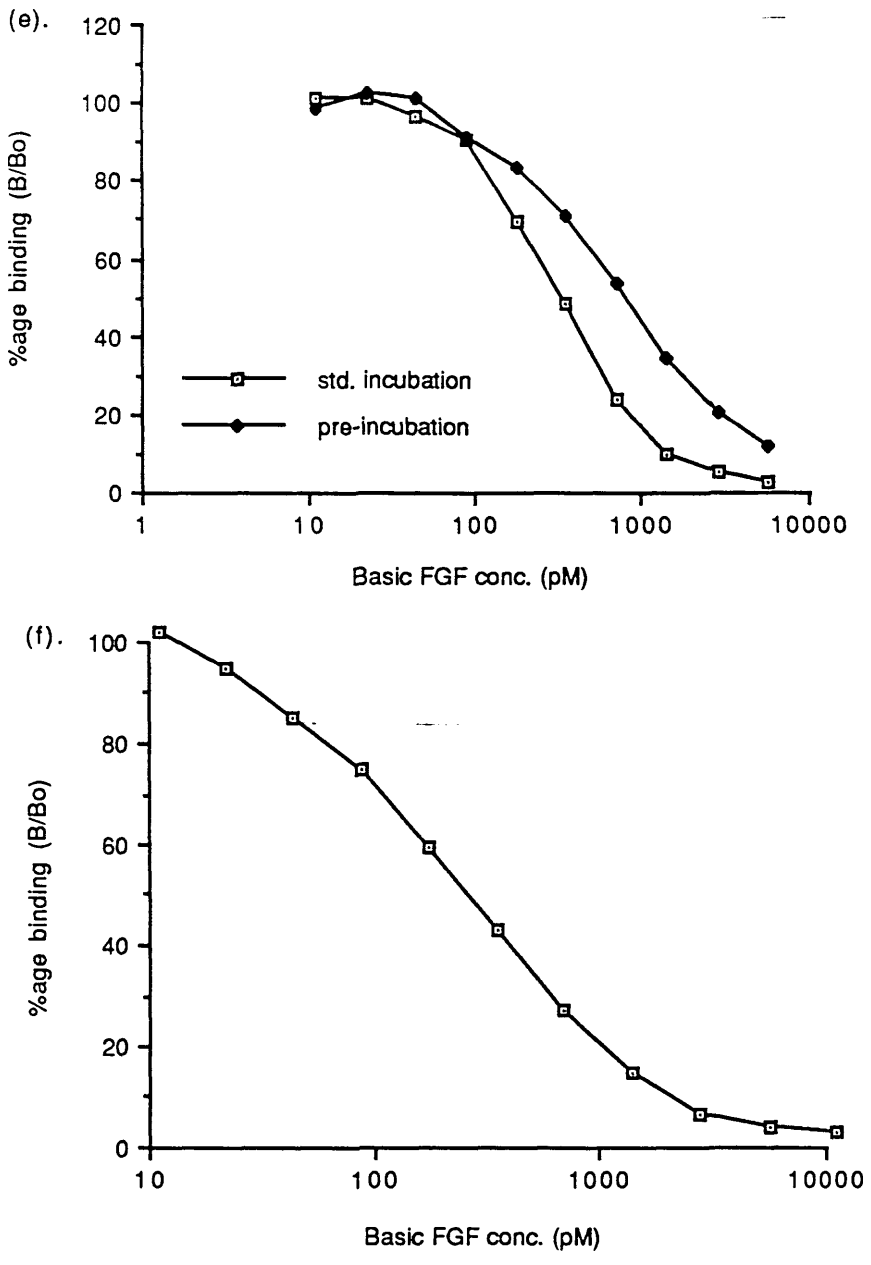


Fig. 3.9 Basic FGF RIA standard curves showing the effect of (a) 1st antibody concentration, (b) label concentration, (c) incubation times, (d) buffer pH, and (e) pre-incubation along with an estimate of assay sensitivity (f)

A pool prepared by spiking assay buffer with 500pM rbFGF was used for assessing assay performance. It was included several times in each assay. The first value obtained in each assay allowed the interassay variation to be calculated as mean/s.d. x 100 (Coefficient of variation or C.V.). The values were 467 ± 80 (mean ± s.d.); n=21; C.V.= 17.1%. The intraassay variation was calculated as the mean value of the variation of the pool within each assay and was 9.6%. The mean percentage total binding in the assays was 24.2%.

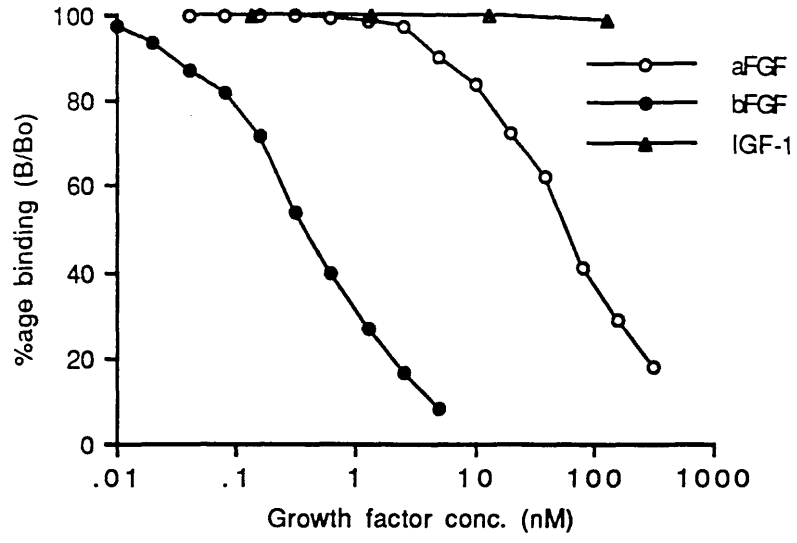


Fig. 3.10 Cross reactivity of aFGF and IGF-1 in the RIA

When samples of BREC conditioned medium were assayed (see Section 6.2.1), standards were diluted in the cell culture medium used and a comparison of the standard curve produced compared with that produced when standards were diluted in FGF buffer is shown in Fig. 3.11.

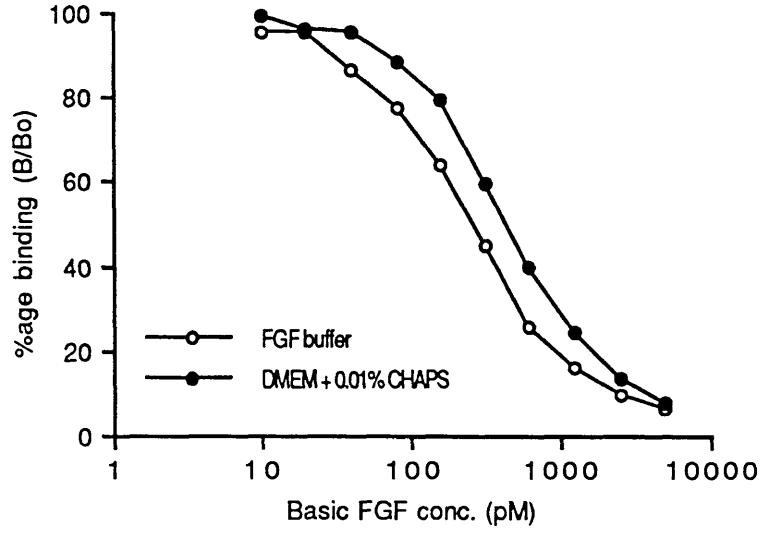


Fig. 3.11 Basic FGF RIA standard curves with standards prepared in DMEM containing 0.01% CHAPS or in FGF buffer

The culture medium used did affect the assay parameters so all assays measuring bFGF in cell conditioned medium used standards prepared in DMEM containing 0.01% CHAPS. A seperate pool was prepared by spiking culture medium with 500 pM rbFGF. Inter and intra-assay variations were calculated as above. Inter-assay pool values were 516 ± 183; n=17; C.V.= 35.5%. Intra-assay variation was 11.0% and the mean

percentage total binding was 31.3%.

3.3.4 Measurement of bFGF in clinical samples

Serum and plasma

Twenty serum samples from both diabetic patients and non-diabetics were assayed in the RIA using standards made up in serum that had been stripped of peptides by mixing overnight with activated charcoal followed by centrifugation and filtration through an 0.2μ filter to remove charcoal particles. All bFGF values were below the sensitivity of the assay (50pM). A subset of these serum samples were compared with EDTA plasma and heparin plasma samples taken from the same patients at the same time. Basic FGF values in EDTA plasma samples were below 50pM except for 3 which were 51, 53 and 65 pM respectively. Heparinized plasma samples however all contained apparent bFGF immunorectivity with values of 357 ± 62 pM (mean ± s.d.; range = 245-452 pM; n=11). To assess wether these measurements represented interference by heparin in the RIA, standard curves were prepared in FGF buffer with and without the addition of 20 μgml^{-1}heparin. The results presented in Fig 3.12 show that heparin shifts the standard curve to the right. The apparent immunoreactivity in the heparinized plasma samples is therefore probably due to heparin competing with the antibody for binding ^{125}IrbFGF.

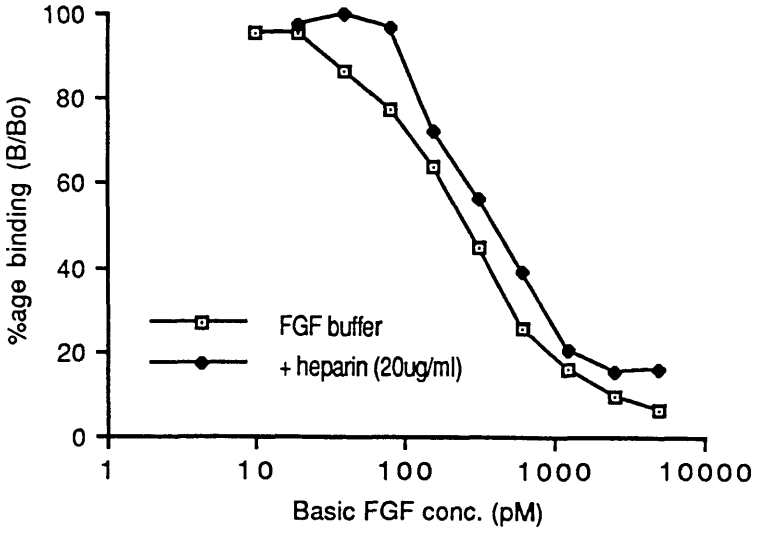


Fig. 3.12 The effect of heparin on the bFGF RIA standard curve

Vitreous

Vitreous and serum samples were obtained from 7 patients at the time of vitrectomy (provided by Moorfields Eye Hospital, London, U.K.). Five patients were diabetic with a history of proliferative diabetic retinopathy and 2 patients were non-diabetic. The samples were stored frozen at -20°C. Radioimmunoassay for bFGF and IGF-1 (see Section 2.4) was carried out on both serum and vitreous samples with standards prepared in stripped serum and bovine vitreous respectively. Basic FGF levels were below the limits of sensitivity of the assay (<50pM) in both serum and vitreous. IGF-1 results were 28.9 ± 4.9 nM (serum) and 3.6 ± 0.9 nM (vitreous) in the diabetic patients and 26.6 nM (serum) and 3.1 nM (vitreous) in the non-diabetics. Results are expressed as mean ± SEM for the diabetic group and mean for the non-diabetics. There was no difference between the groups or correlation between serum and vitreous values.

3.3.5 Measurement of bFGF in retinal extracts

The bFGF content of three batches of fresh bovine retinal extract measured by RIA was 156, 1040 and 5120 pM. When an investigation of the release of this growth factor with time was carried out the results (Fig. 3.13) showed a rapid increase from 2 hours onwards and as the length of retinal incubation when producing the extracts was not strictly controlled this could account for some of the variability in retinal extract bFGF immunoreactivity.

The stability of bFGF immunoreactivity in these extracts could also be involved in the variable concentrations. The extracts were stored aliquoted at -20°C and the change in bFGF immunoreactivity with time was measured. The results are shown in Fig. 3.14.

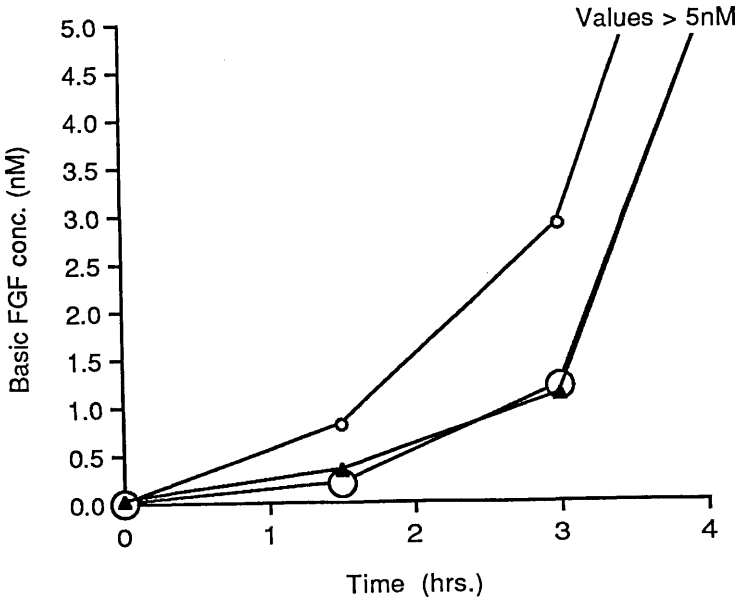


Fig. 3.13 Basic FGF immunoreactivity released from bovine neural retinae with time

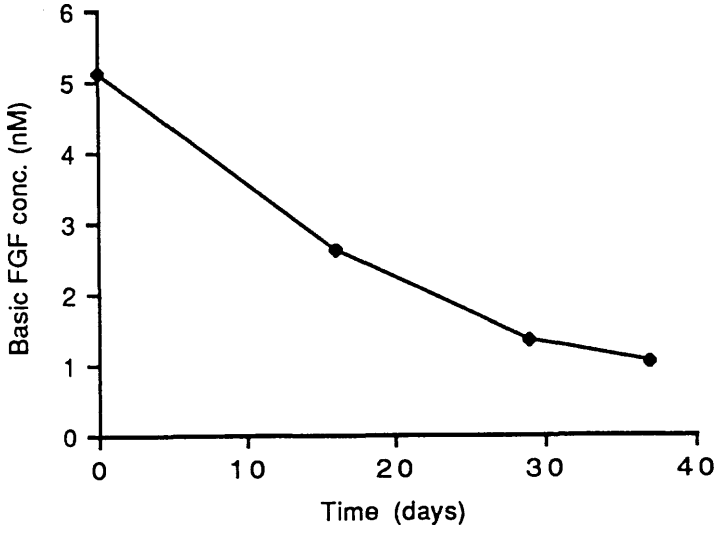


Fig. 3.14 Stability of bFGF immunoreactivity in bovine retinal extracts stored at -20°C

The identity of the bFGF immunoreactivity in the retinal extracts was shown by parallelism in the RIA shown in Fig. 3.15 and by heparin-Sepharose chromatography.

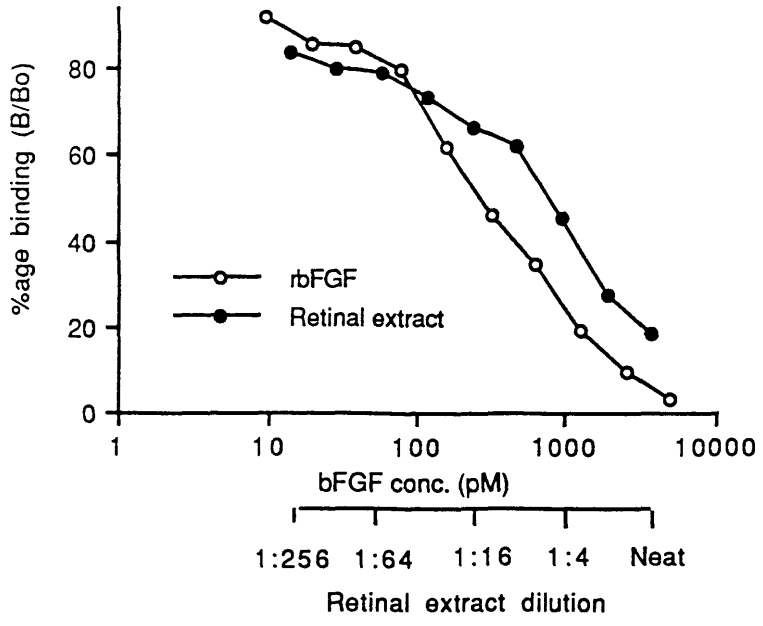
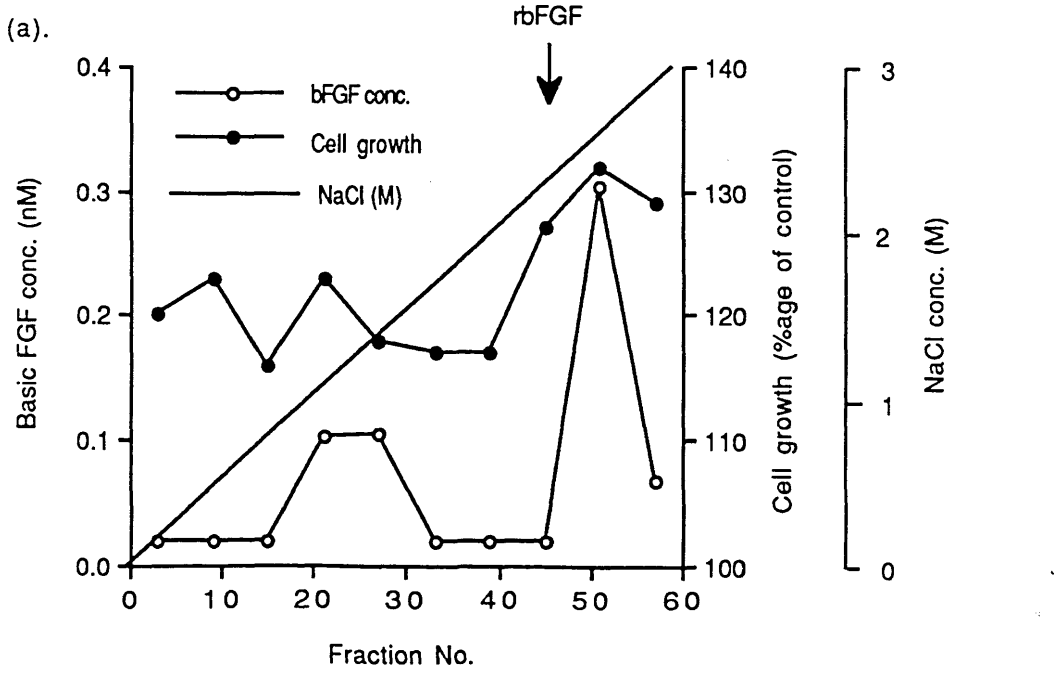


Fig. 3.15 Comparison of the ability of doubling dilutions of retinal extract and rbFGF to compete with ^{125}IrbFGF for binding to anti-bFGF antibody

The heparin-Sepharose elution profiles of two batches of retinal extract are shown in Fig. 3.16. Bioassay results obtained using the Coomassie blue staining method are shown for the fractions of the first elution series and protein determinations for the fractions of the second.

Fig. 3.16a



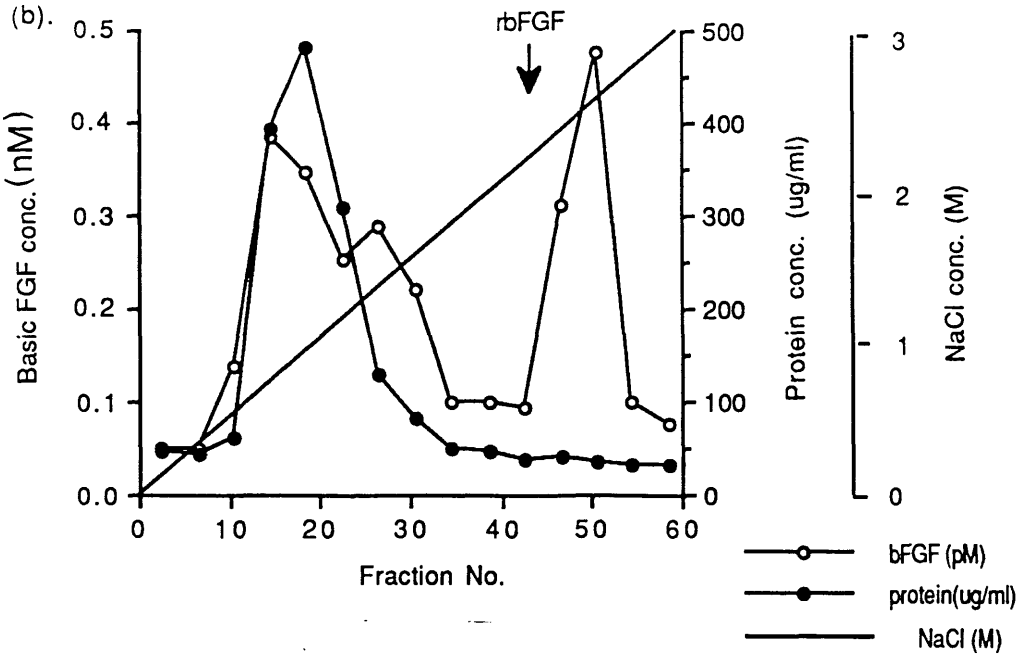


Fig. 3.16 Heparin-Sepharose elution profiles of two batches of retinal extract. (a) Batch 1, each point is from a pool of 6 fractions and bFGF immunoreactivity and cell growth are shown; (b) Batch 2, each point is from a pool of 4 fractions showing bFGF immunoreactivity and protein concentration. The point where rbFGF was eluted from a heparin-Sepharose column is shown in each case (see Fig. 6.1)

The elution profiles of the retinal extracts both show two main peaks of bFGF immunoreactivity. In batch 1 the first peak begins at 0.8M NaCl and has a mid point at 1.2M NaCl. In batch 2 the first peak is broad beginning at a NaCl concentration of 0.3M, reaching its highest point at 0.7M with a midpoint at 1.0M. The second peak was eluted at NaCl concentrations begining at 2.3M and 2.1M and peaking at 2.6M and 2.5M in batches 1 and 2 respectively. The second peaks were eluted at a NaCl concentration similar to that where rbFGF was eluted (peak at 2.2M) and indicate the identity of these peaks with bFGF. In batch 1 the bioactivity of the fractions shows a peak corresponding to the peak of bFGF immunoreactivity eluted at the higher NaCl concentration. In batch 2 the protein concentration of the fractions produce a peak which overlaps the peak of bFGF immunoreactivity eluted at the lower NaCl concentration. The bFGF and protein

concentration of the fractions which passed through the column when the sample was loaded were also measured in batch 2. Basic FGF immunoreactivity reached a peak of 290 pM with a corresponding protein concentration of 518 μgml^{-1}.

3.4 Sandwich enzyme-linked immunosorbent (ELISA) assays for bFGF

3.4.1 Assay based on the affinity of bFGF for heparin

A typical standard curve using rbFGF is shown in Fig. 3.17. The minimum detectable concentration of bFGF using this assay was 8pM

Measurement of bFGF in BREC conditioned medium

Medium conditioned by BREC, containing measurable concentrations of bFGF in the RIA were included in this assay. Values were below the limit of detection (8pM).

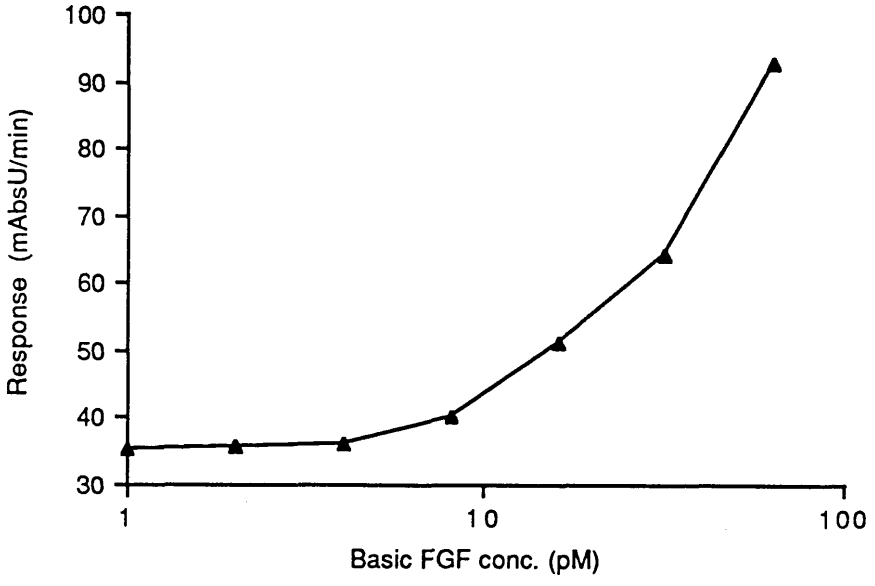


Fig. 3.17 Heparin-based ELISA standard curve using rbFGF.

This assay was similar to that used by Sato et al.,(1986a) to measure bFGF release from bovine corneal endothelial cells and human astrocytoma cells. To test

whether our assay would produce similar results, an experiment was set up to compare the release of bFGF from BREC and from astrocytoma cells seeded at both high and low density.

Experimental procedure

Bovine retinal endothelial cells and astrocytoma cells were seeded at high density, (3.5×10^4 cells/cm^2) and (2.8×10^4 cells/cm^2) respectively and low density, (1.1×10^4 cells/cm^2) and (9.4×10^3 cells/cm^2) in 25 cm^2 tissue culture flasks. After overnight attachment the cells were washed with 2ml of HBSS per flask and 1ml of DMEM containing 0.1% BSA was added to each flask. DMEM containing 0.1% BSA was also added to confluent cultures of BREC to prepare medium for the dilution of standards. After 18hrs. the medium was removed, centrifuged at 925g for 10mins and stored frozen at -20°C prior to assay in the ELISA.

Results

All samples were below the limit of detection of the assay (8pM).

3.4.2 ELISA using anti-bFGF monoclonal antibody

A sandwich ELISA assay using anti-bFGF monoclonal antibodies to coat the plate was set up in order to improve the specificity of the assay and prevent interference from other heparin-binding proteins present in the samples. Initial results with this assay produced uniformly high backgrounds. A test assay was carried out to determine whether rbFGF was able to bind to anti-bFGF monoclonal antibodies coating the plate using ^{125}I labelled rbFGF. The results are shown in table 3.5. Non-immune mouse serum (1:25) was used to coat the wells for measurement of non-specific binding.

Coating Conc. (μg/ml)	NSB	Monoclonal Antibody			
		FE8	DE6	DG2	AF11
5.0	96±37	6554±115	5456±586	1224±277	6483±30
20.0	124±23	7106±58	7024±133	3153±217	6970±79

Table 3.5 Binding of ^{125}I-rbFGF to PVC plates coated with anti-bFGF monoclonal antibodies. Results are expressed as cpm bound per well (mean ± s.d.; n=3)

It was evident from these results that rbFGF bound to the coating antibodies and that an antibody concentration of 20μgml^{-1} increased the amount of binding. The antibody DG2 appeared to be less effective at binding rbFGF. It was also realized that because the 2nd antibody in the ELISA (anti-bFGF1-24) had been raised against a peptide-BSA conjugate it would contain immunoglobulins directed against BSA therefore the use of BSA to block non-specific binding to the plates could be responsible for the uniformly high backgrounds obtained in the ELISA. A range of other blocking agents was tried and 5% w/v Marvel dried milk powder was the most effective at reducing background. Using 5% Marvel as the blocking agent an ELISA was set up to compare the optical density in wells incubated with 4nM rbFGF compared to control wells for each of the four monoclonal antibodies and NIMS (1:25). The effect of preincubating the bFGF1-24 antibody with 0.5% w/v BSA for 2hrs prior to addition to the wells was also tested. Colour development was stopped after 3mins by the addition of 50μl of 2M HCl per well. The results are shown in table 3.6 as the mean optical density in duplicate wells.

Blocking Agent	Monoclonal Antibody								
	bFGF	+	-	+	-	+	-	+	-
	NIMS	AF11		FE8		DG2		DE6	
5% Marvel	.122	.530	.184	.526	.164	.491	.216	.483	.237
+Preincubated 2nd Antibody	.098	.601	.108	.616	.108	.500	.107	.558	.107

Table 3.6 Comparison of coating antibody, 2nd antibody treatment and bFGF (4nM) on optical density in the ELISA

The results show that the ELISA is capable of detecting rbFGF. It is also clear that preincubating the 2nd antibody with BSA reduces the background and that AF11 and FE8 are the most effective antibodies.

Using these results standard curves were produced to investigate the detection limits of this assay. A typical standard curve is shown in Fig. 3.18. The detection limit is 2pM which represents a significant improvement over the heparin based ELISA and a 10 fold increase in sensitivity over the RIA.

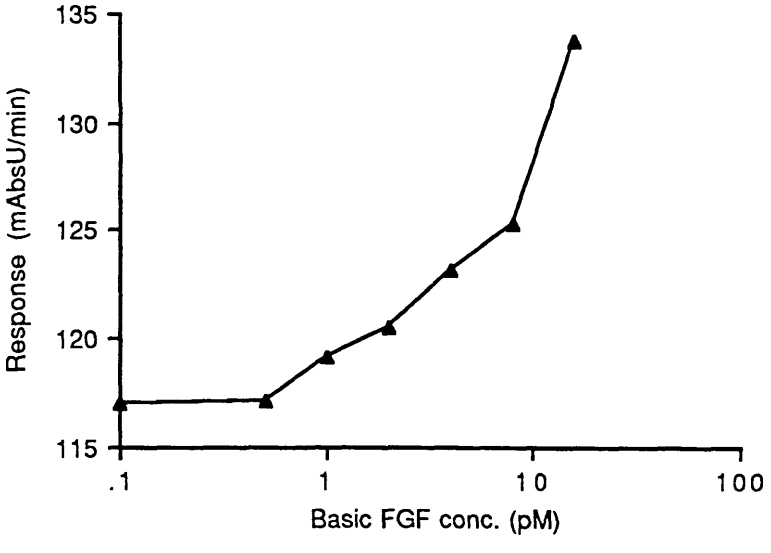


Fig. 3.18 Basic FGF monoclonal antibody ELISA standard curve

3.4.3 Basic FGF in vitreous samples

Bovine vitreous

Bovine vitreous showed measurable bFGF immunoreactivity with a value of 4.4 pM. Standards prepared in bovine vitreous were compared with those prepared in FGF buffer containing 0.2% gelatin and the results showed a shift in the standard curve (Fig.3.19) again indicating the presence of bFGF in the vitreous or interference in the assay.

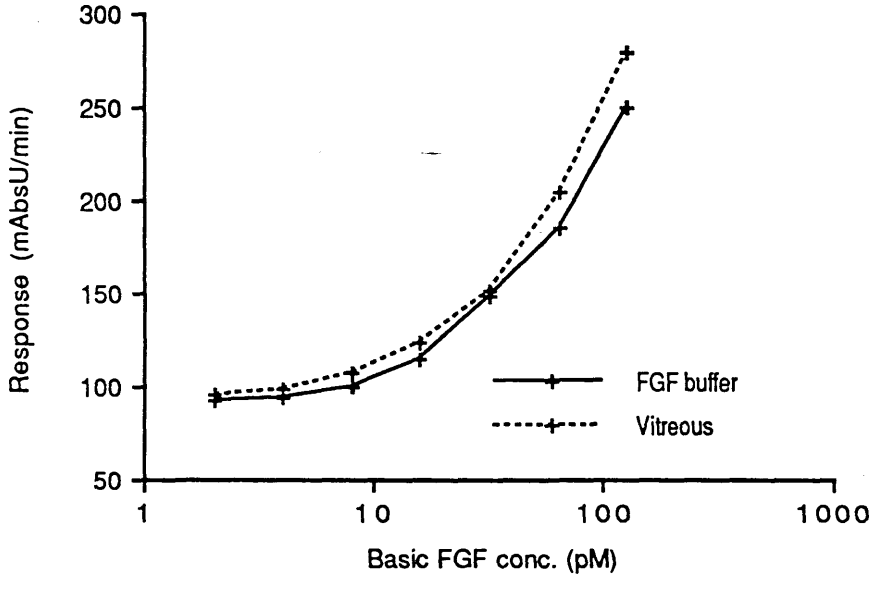


Fig. 3.19 Effect of bovine vitreous on the ELISA standard curve

Human and animal vitreous

Although bFGF was not detected in human vitreous samples using the RIA (Section 3.2.4) because of the increased sensitivity of the monoclonal antibody ELISA a new group of human vitreous samples and vitreous obtained from experimental animals were assayed for bFGF using this method.

Method

Vitreous samples were obtained during vitrectomy from diabetic patients with a history of proliferative diabetic retinopathy (provided by Moorfields Eye Hospital). Vitreous

samples were also obtained from two miniature pigs and one rhesus monkey sacrificed after retinal blood flow studies and from bullock eyes used for the isolation of retinal microvessels. The monkey had one eye laser-treated prior to enucleation. The samples were freeze dried and stored at -20°C before use. They were reconstituted to their original volume with distilled H_2O and 100μl was assayed in duplicate in the monoclonal antibody ELISA against rbFGF standards made up in reconstituted bovine vitreous. Albumin levels were measured in each sample using an immunoturbidometric albumin assay on a Technicon RA1000 Autoanalyser (Technicon, Basingstoke, U.K.).

Results

Basic FGF levels in each sample as determined from the standard curve are shown in Table 3.7 alongside albumin levels

Sample	mAbsU/min	bFGF (pM)	Albumin (mg/l)
Patient 1	21.98	18	74
Patient 2	106.5	>128	>300
Patient 3	115.1	>128	267
Patient 4	30.9	43	>300
Patient 5	474.5	>128	>300
Pig 1	16.33	<2	<2
Pig 2	17.62	<2	<2
Pig 3	16.31	<2	<2
Pig 4	15.83	<2	<2
Monkey+Laser	15.77	<2	14
Monkey-Laser	84.17	>128	51

Table 3.7 Basic FGF and albumin levels in vitreous samples

The initial results suggested that bFGF was present in the vitreous samples from patients with proliferative diabetic retinopathy however it was realized that the highest bFGF values occured in the samples visually contaminated with blood. The albumin values confirmed a correlation between bFGF concentration and the presence of serum proteins in the samples. The likely explanation for the apparent bFGF measurements in the samples is non-specific binding of serum albumin in these samples cross-reacting with the bFGF 1-24 antibody.

3.5 Immunoblotting

Immunoblotting was carried out to determine whether both bFGF and aFGF were present in bovine vitreous samples. Freeze dried bovine vitreous was reconstituted in distilled water. The results are shown in Fig. 3.20.

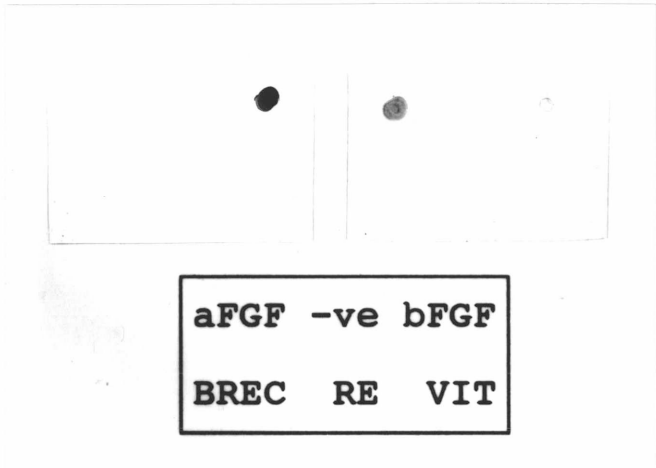


Fig. 3.20 Immunoblotting of bovine vitreous. The left hand blot was incubated with anti-bFGF monoclonal antibodies and the right hand blot with anti-aFGF polyclonal antibodies. BREC = BREC conditioned medium, RE = retinal extract and VIT = bovine vitreous

Both immunoblots show appropriate staining for aFGF and bFGF respectively with no staining of DMEM containing 10% v/v tryptose phosphate broth culture medium. Both medium conditioned by BREC and retinal extracts show the presence of

both acidic and bFGF although there is some crossreactivity of the anti-aFGF antibody with bFGF and this may account for the apparent aFGF immunoreactivity in these samples. Bovine vitreous shows no staining for aFGF and a very weak positive reaction with anti-bFGF antibody.

3.6 Discussion

Polyclonal antibodies were successfully raised against the N-terminal 1-9 amino acids of $bFGF_{1-146}$. The relatively low titre obtained, (1:2000, producing 40% binding) demonstrates the problems in obtaining a high titre, specific antiserum against a small peptide. The peptide itself has little immunogenicity and must be conjugated to a larger protein. The antibodies generated are polyvalent and a proportion will be directed against antigenic determinants on the carrier protein. A higher titre would have been expected if polyclonal antibodies had been raised against the whole bFGF molecule. The difficulty in choosing an appropriate amino acid sequence of the molecule against which to raise antibodies became apparent during the development of the RIA. The N-teminal peptide of bFGF was chosen because its sequence was known and it was unlikely to be a hidden epitope within the tertiary structure of the protein. It soon became clear however that the N-terminal was a variable region of bFGF with extended and truncated forms being described (Ueno et al., 1986). We decided that antibodies directed against this part of the molecule would still be useful for quantitating bFGF in vitreous and retinal samples because the truncated variety had not been found in retinal tissue (Baird et al., 1985a). It is still not clear whether the production of truncated forms is tissue specific although the cleavage of $bFGF_{1-155}$ to $bFGF_{1-146}$ (See Fig. 1.4) can be prevented by isolating bFGF in the presence of protease inhibitors and all truncated forms may be an artifact of the isolation procedure.

A more fundamental problem with the assay was the discovery of an aminopeptidase present in serum and tissue extracts that cleaved the Pro-Ala-Leu epitope on Tyr^{10}(FGF)1-10 recognized by the antibody (Gauthier et al., 1987). We

were unable to inhibit this enzyme sufficiently with protease inhibitors and use of the RIA based on this peptide and antibodies raised against it was discontinued.

The second RIA developed for bFGF used an antibody which was also raised against an N-terminal petide sequence. This antibody recognized an epitope comprising amino acids 11-15 of $bFGF_{1-146}$ (Baird & Ling, 1987). This epitope was not affected by the aminopeptidase. The 1-15 truncated variants of $bFGF_{1-146}$ would not be recognized by the antibody but, as discussed above these were not present in retinal tissue extracts. The sensitivity of the RIA (18pM) approaches the maximum sensitivity of this technique (about 1pM) and indicates antibodies with high binding avidity. The optimization procedures also indicated a reasonably robust assay. The main difficulty with the assay was working with low concentrations of bFGF which was extremely adherent due to its cationic and hydrophobic nature. It was therefore stored aliquoted at the highest possible concentration for standards and pools. Basic FGF labelled with ^{125}I also has a high affinity for BSA (Neufeld & Gospodarowicz, 1985) and therefore 0.2% w/v gelatin was used as a carrier protein.

The chloramine-T method was used to iodinate rbFGF and presented no particular problems even though it is generally considered to use harsh reaction conditions. A label of high specific activity could be prepared because it was repurified before use and this contributed to the sensitivity of the assay.

Our inability to detect bFGF in human serum samples is in agreement with most other investigators. Some early reports of bFGF levels in serum (Baird et al., 1986) were made using an RIA based on anti-bFGF1-10 antibodies and cannot now be considered reliable. It has since been reported that bFGF is present in the serum of some patients with familial multiple endocrine neoplasia (Zimering et al., 1990) and a recent study using an enzyme immunoassay with a sensitivity for serum samples of 2pM reported levels of bFGF between 2 and 12.5pM in 16 out of 51 samples of serum from normal human volunteers (Watanabe et al., 1991). The discrepancy between the latter results and those of previous studies could be due

to the specificity of the assay which used anti-bFGF monoclonal antibodies and was not effected by inteference from heparin, heparan sulphate or heparin binding proteins. Basic FGF would however be unlikely to circulate in an active form because of its potent actions on vascular cell proliferation and it has been suggested that binding to α_2-macroglobulin could rapidly remove any bFGF that was released into the circulation (Dennis et al., (1989). Whether the bFGF detected in the above study is bioactive remains to be determined.

Our inability to detect bFGF in the vitreous of diabetic patients with a history of proliferative retinopathy is in contrast to the study by Sivalingam et al., (1990). They used a two-site ELISA with a sensitivity of 146 pM, but they also diluted the samples 1:10 so the minimum detectable limit was 1500 pM. This compares with a sensitivity for our RIA of 18 pM. In the study by Sivalingham et al., (1990), half of the vitreous samples from patients with proliferative diabetic retinopathy had undetectable bFGF and they correlated measurable bFGF levels with the presence of active proliferation at the time of vitrectomy. It is possible that vitreous samples from some patients with proliferative diabetic retinopathy have levels of bFGF in the vitreous below 18 pM and that the small number of vitreous samples examined by us were all from a subset with inactive proliferative diabetic retinopathy. It is also possible that differences between the assays accounted for our inability to detect bFGF in vitreous. The standardisation of assays for bFGF between laboratories has not yet been addressed, nor has the different types of assay used. Thus different bFGF standard preparations and antibodies used by different groups may lead to large variations in bFGF measurement.

We were not able to show increased IGF-1 in the vitreous of diabetic patients compared to non-diabetic controls, unlike a previous study (Grant et al., 1986). This group also showed a correlation between levels of IGF-1 in the serum and in the vitreous suggesting vascular leakage as the source of the growth factor. The small number of samples assayed by us could account for this discrepancy as could differences in

assay protocols. The levels of IGF-1 in the vitreous measured by us were higher than those obtained by Grant et al., (1986). This could be due to differences in the extraction and processing of the samples. We used neat vitreous whereas Grant and co-workers collected the vitreous in buffer and subsequently dialysed the samples prior to reconstitution and assay, a process which they found decreased the IGF-1 concentration by at least 50%.

The ELISA assays we have developed for bFGF, whilst not as well characterised as the RIA, gave reproducible standard curves using rbFGF as the standard and were more sensitive than the RIA. Using the heparin-coated plate ELISA however, we could not detect bFGF in bovine retinal endothelial cell conditioned medium or medium conditioned by a human astrocytoma cell line in contrast to the results of Sato et al., (1989a) who showed a concentration of 30 fmol/10^5cells in medium conditioned by the same astrocytoma cell line and 0.4 fmol/10^5cells in medium conditioned by bovine corneal endothelial cells. If our astrocytoma cultures produced a similar amount of bFGF to that seen by Sato et al., then we should have been able to detect this using the heparin-based ELISA. The human astrocytoma cells exist as two distinct phenotypes (see Fig. 4.8) and this could provide a possible explanation for our different results. The long spindle shaped cells produce bFGF whereas the flat polyogonal shaped cells do not (Murphy PR, personal communication) and only cultures containing predominantly spindle shaped cells were used to produce conditioned medium in the study by Sato and co-workers whereas we produced conditioned medium from heterogenous cultures.

The two-site ELISA developed using anti-bFGF monoclonal antibodies to coat the plate also produced a standard curve with an improved limit of detection over the RIA. The assay had the drawback of using the polyclonal antibody raised against a peptide-albumin conjugate to complete the sandwich. This antibody cross reacted with serum albumin and prevented BSA being used to block non-specific binding to the plate. The use of low-fat milk powder as an alternative blocking agent was successful in preventing the non-specific

adsorption of bFGF but not serum albumin from samples. This invalidated the measurements of bFGF in vitreous samples from diabetic patients because these samples were contaminated with serum proteins originating from either the surgical procedures or from a previous vitreous haemorrhage. The detection of bFGF in bovine vitreous using the monoclonal antibody-based ELISA and the effect of bovine vitreous on the ELISA standard curve (see Fig 3.19) could be explained by the presence of albumin in these samples. However, the immunoblotting results using anti-bFGF monoclonal antibodies provide evidence for the presence of bFGF, though not aFGF, in normal bovine vitreous. It is difficult to explain the presence of this potent mitogen in normal bovine vitreous although one possible reason could be that it was released from the bovine neural retina after death and enucleation following post-mortem changes to this tissue.

High levels of bFGF immunoreactivity were present in the retinal extracts. This is in accordance with previous work showing both basic and aFGF in extracts of this tissue. Several of these studies used the effects of FGF on cell proliferation and its elution profile from heparin-Sepharose to characterize the forms present (Courty et al., 1985; Baird et al., 1985a). Heparin-Sepharose profiles of retinal extracts produced by us showed two peaks of immunoreactivity which were eluted at salt concentrations characteristic of acidic and bFGF. The second of the two peaks was eluted at a higher salt concentration than rbFGF and that seen for bFGF by other investigators (Klagsbrun et al., 1987) however, it is known that different batches of heparin-Sepharose vary in their retention of heparin-binding growth factors (Gospodarowicz, 1987). The fractions of this second peak were able to stimulate the proliferation of BREC however those of the first peak were not. The peak of bFGF immunorectivity that was eluted at this low salt concentration could be due to aFGF cross-reacting in the RIA. However, because of the low cross reactivity of this protein in the RIA it would indicate very high concentrations of aFGF in the retinal extracts and the high protein concentrations present in these fractions would support this idea. Although immunoblotting

with anti-aFGF antibodies showed that aFGF is present in the retinal extracts (see Fig. 6.2) it is unlikely to be at high enough concentrations to be responsible for the peak of immunoreactivity seen in the heparin-Sepharose profiles. An alternative explanation was provided by Baird et al., (1985c) who reported the elution of some bFGF at low salt concentrations. The high protein content of these fractions could be due to the presence of weak heparin-binding factors and these could include inhibitory factors. This would provide an explanation for the lack of BREC proliferation in response to the fractions eluted at low salt concentrations.

Chapter 4. GROWTH AND CHARACTERIZATION OF BREC AND BRP IN CELL CULTURE

4.1 Introduction

A reliable and reproducible method for the culture of retinal vascular cells was required in order to investigate the production of bFGF by these cells and their response to this and other growth factors. Methods for retinal endothelial cell culture using added bFGF and aFGF or retinal extracts and endothelial cell growth supplements containing these factors have been used successfully (Gitlin & D'Amore, 1983; Gospodarowicz et al., 1986b). However, we did not wish to use these supplements because they could interfere with the endogenous production or response to bFGF during experiments.

Two methods were investigated for the isolation and culture of BREC and BRP and their relative merits will be discussed. As already described there are several cell types present in the retina which could contaminate the cultures. It was therefore necessary to develop methods for specifically characterizing the cells and ensuring that their differentiated phenotypes were maintained throughout the passages used in experiments.

The successful culture of retinal endothelial cells in the absence of added growth factors requires a substrate of ECM or one of its components. The response of retinal capillary endothelial cells to added bFGF has been shown to be affected by the substrate on which they were grown (Gospodarowicz et al., 1986b). We therefore compared the ability of three commonly used substrates, gelatin, collagen and fibronectin to maintain the growth of BREC and examined their effects on the proliferative response to bFGF.

Cell freezing is an essential technique to maintain stocks of cells for experimental procedures and particularly to enable experiments to be carried out on several batches of cells at the same time. Batches of cells of the same passage number were frozen for this purpose and the viability of frozen cells after thawing and their ability to respond to

bFGF was assessed. Finally Millicell culture plate inserts were used as a potential means of studying the release of growth factors from the basal and abluminal cell surface.

4.2 Methods of retinal cell culture

4.2.1 Microvessel isolation

Bullock eyes

The bullock eyes were obtained on a weekly basis from a London slaughter house and were of a similar size both within and between batches. They were kept at 4°C until use and could be kept at this temperature for up to 28 hrs without any noticable change in the growth of the isolated cells. Only slight variations were seen in the growth of cells from different batches of eyes although occasionally cell growth was very poor. Whether this was due to a property of the group of animals from which the eyes were taken or to methodological errors in the isolation and growth of the retinal cells is not clear. It was evident however that cell growth was particularly poor from microvessels isolated from calves eyes. These eyes were noticably smaller than their bullock counterparts and they were not used once the problem was discovered.

Methods of microvessel isolation

The two methods described in Section 2.5.1 for the isolation of retinal microvessels both produced vessel fragments from which retinal endothelial cells and pericytes grew. The method based on that of McIntosh et al., (1988) produced the best yield because it produced frament of all the retinal vessels, both large and small, whereas the method of Wong et al., (1987) was for the isolation of capillaries only. It was found empirically that the use of differential enzyme treatment time, as employed in the Wong method, improved the homogeneity of the cell type in primary culture. BRP grew well from vessels treated for 65mins whereas

endothelial cells grew better when incubation with collagenase-dispase was for 90mins. The density at which the microvessels were seeded also influenced the purity of the primary culture. The most homogenous cultures of BRP were produced when vessel fragments obtained from 3 bullock eyes were seeded per 75 cm^2 flask while the most appropriate seeding concentration for BREC was vessel fragments from 5 bullock eyes per 75 cm^2 flask.

4.2.2 Growth and subculture of cells

Bovine retinal pericytes

Bovine retinal pericytes grew slowly in primary culture and when the above seeding density was used, reached confluence in 11 days. There was a tendency in the primary cultures for an initial growth of large endothelial islands contaminating the pericyte cultures particularly when the microvessels were seeded at a higher density. These islands of endothelial cells grew strongly at first but after 3-4 days began to show a brighter appearance with phase contrast microscopy because the islands were contracting and not adhering as strongly to the substratum. If left, the cells in these islands eventually died and pericyte growth continued. The islands could also be removed by a process of differential trypsinization (see Section 2.5.2). Confluent cultures of BRP did not show contact inhibition and the cells overgrew, forming mounds of cells.

BRP were initially characterised by their distinctive morphological appearance. They have an irregular periphery with numerous cytoplasmic processes as shown in Fig. 4.1.

They showed no immunoflourescent staining for Factor VIII-related antigen but stained positively for 3G5, a ganglioside antigen which in the retina is specific for pericytes (Nayak et al., 1988) (Fig 4.2).

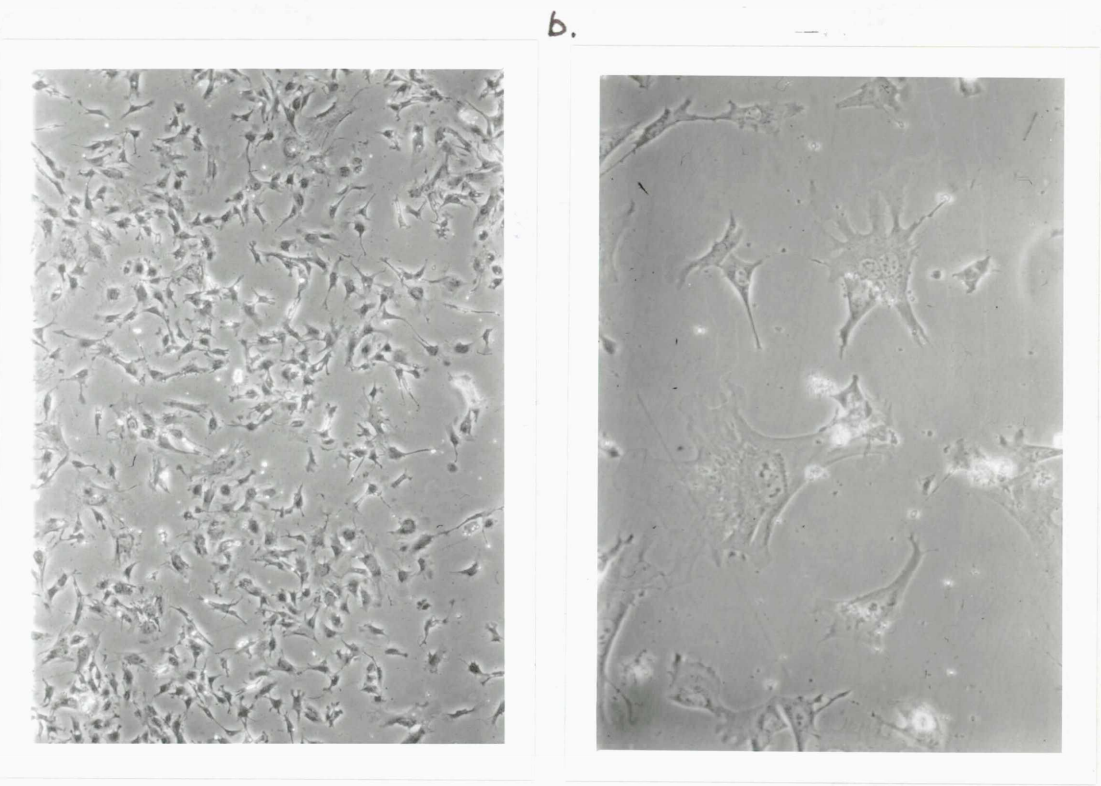


Fig 4.1 First passage bovine retinal pericytes in culture (a) x 40 and (b) x 200

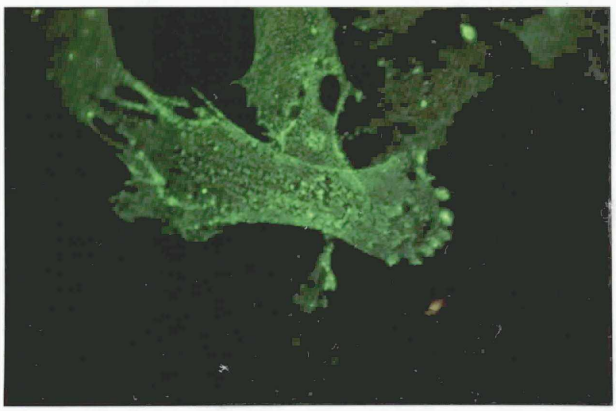


Fig 4.2 Immunoflourescent staining of BRP with anti-3G5 antibody

The subculture of BRP was carried out before the cells became overconfluent. The trypsinization time varied between batches of cells but 10-15mins was required in order to detach most cells from the substrate. First passage pericytes retained their morphology and were visually a pure culture. Subsequent passages showed a decrease in the differentiated characteristics of the cells with loss of cytoplasmic processes and an increase in cell size. Some batches of BRP

became growth arrested in second or third passage and replacement of the medium with fresh DMEM containing 20% FCS would not stimulate further growth. For these reasons experiments were carried out on first passage cells although pericyte conditioned medium for the growth of BREC (see below), was produced from second passage BRP.

Bovine retinal endothelial cells

Two methods were tested for their ability to maintain BREC growth in routine culture. The first method based on that of Wong et al., (1988) met with only limited success. Two major problems were encountered with this method, 1. the microvessels did not reliably adhere to the gelatin substrate and 2. BRP conditioned medium often produced no growth stimulation and could inhibit BREC growth. The production of conditioned medium was time consuming and required a lot of pericytes.

The second method based on that of McIntosh et al., (1988) was more successful. Vessel fragments readily adhered to the fibronectin substrate and within 24 hours endothelial cells began growing from these fragments (Fig. 4.3).



Fig. 4.3 Growth of BREC from a retinal vessel fragment after 24hrs in culture. (x 40)

Bovine REC grown from vessel fragments seeded at standard density grew to confluence in 4 days and did not require a change of culture medium. Bovine RP contaminated these primary cultures. However, due to their much slower growth under BREC growth conditions, their numbers were few. The majority of contaminating pericytes were removed on subculture.

Bovine REC had a distinctive morphology growing as islands of closely packed cells with smooth boundaries which did not overlap giving rise to a typical 'cobblestone' appearance (Fig. 4.4).

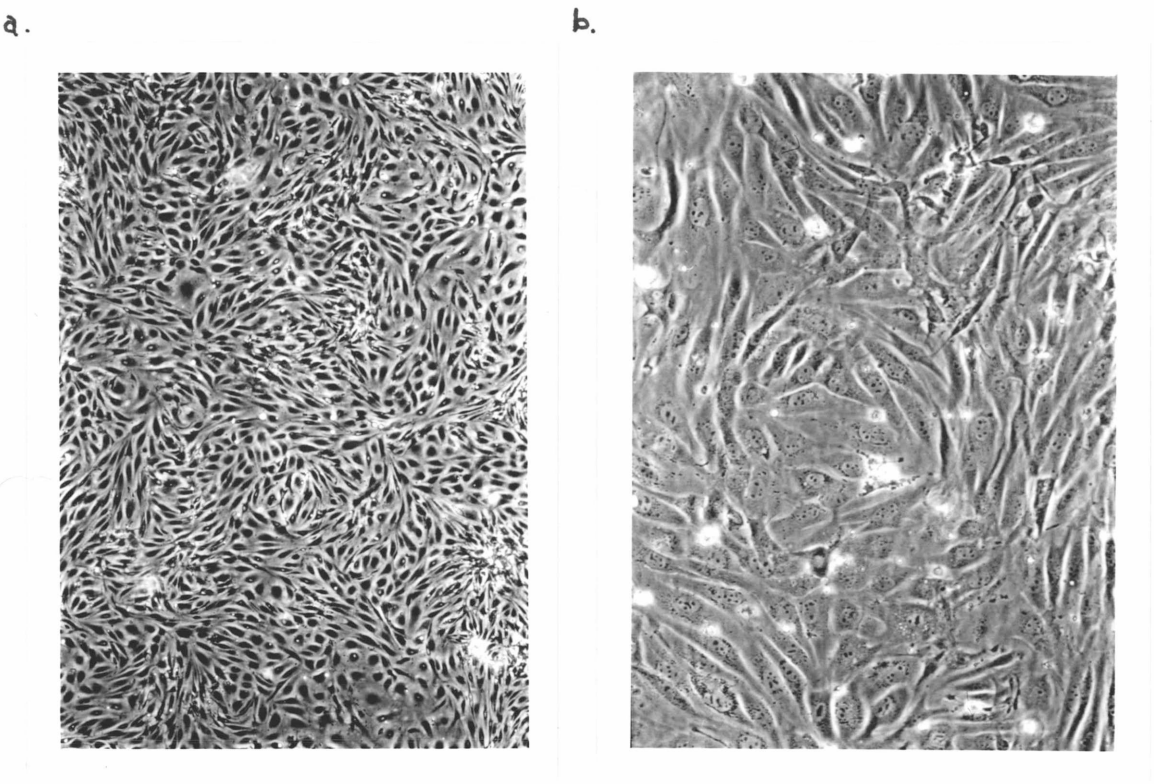


Fig. 4.4 Confluent primary culture of bovine retinal endothelial cells (a) x40 and (b) x200

These cells were further characterized by positive immunoflourescent staining for Factor VIII-related antigen (Fig 4.5)

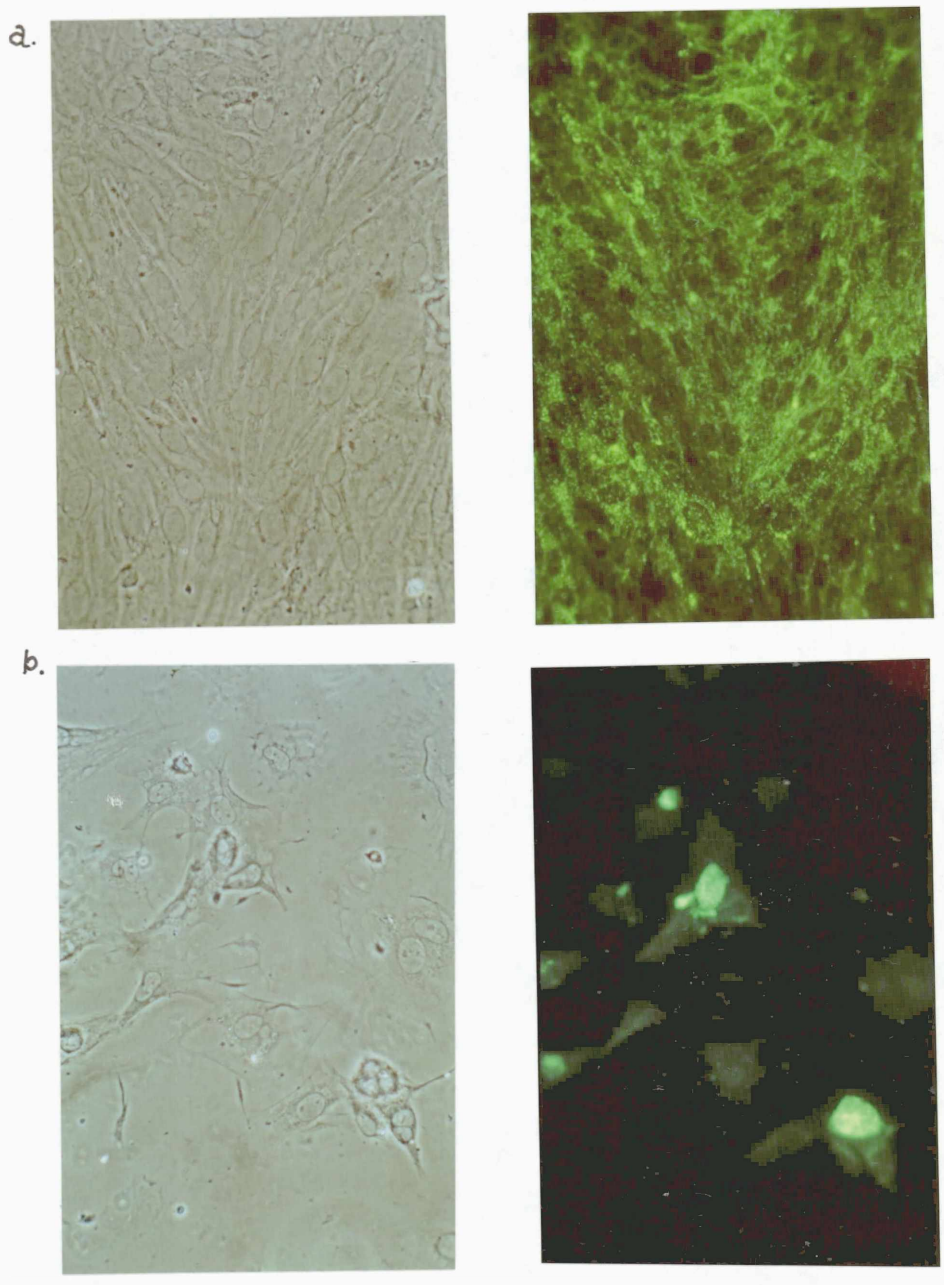


Fig 4.5 (a) Immunoflourescent staining of BREC with antibody to Factor VIII-related antigen (b) Negative staining of BRP. A phase contrast micrograph of the same field of view is shown in each case (x100)

BREC were passaged before they reached confluence because if trypsinization was carried out on confluent cells they subsequently grew less well. Endothelial cells detached from the substrate within 90 seconds and the more strongly adherent pericytes could be left behind. This increased the purity of the cultures to greater than 95%. BREC could be passaged up to 13 times, splitting the cells 1:2 on each

occasion, before they were unable to divide futher. From passage 8 onwards the cells maintained their cobblestone morphology but appeared less substantial under phase contrast becoming more transparent with thinner cell boundaries. Cells at passage 6 still stained positively for Factor VIII-related antigen.

When BREC reached confluence, there was no evidence of contact inhibition and quiescence, the cells continued to divide and packed more tightly into the available growth area. There was also evidence that cells were ejected from the monolayer. Post-confluent cultures showed increased proliferation of contaminating pericytes which formed localized nodular areas on the surface of the endothelial cells. There was also evidence of sprouting (Smith, 1989) beneath the endothelial cell monolayer.

The growth of BREC in culture was investigated using Coomassie blue staining to measure cell growth (see Section 2.6.1). After attachment, and each day for 3 days, wells were stained with Coomassie blue and the optical density measured against a blank containing Coomassie blue alone. The results are shown in Fig. 4.6

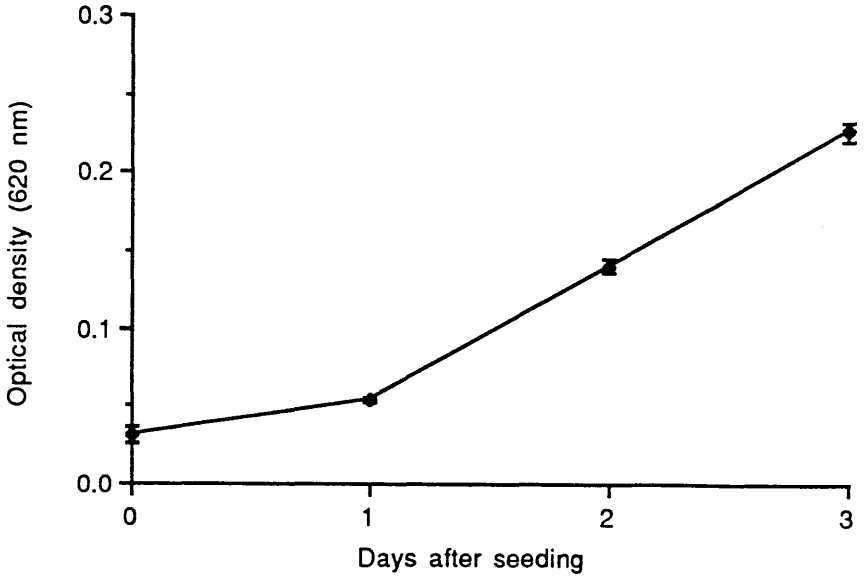


Fig. 4.6 BREC proliferation as measured by mean daily increase in optical density ± SEM at 620nm following Coomassie blue staining (n=10)

The cells became visibly confluent after 48hrs, however the increase in absorbance was linear up to 72hrs. These results confirm the visual evidence that BREC did not become quiescent upon reaching confluence.

4.2.3 Comparison of BREC growth on different substrates

An experiment was carried out to determine which substrate best supported BREC proliferation and whether the substrate affected the proliferative response of BREC to bFGF.

Method

Two wells of a 6-well cell culture plate were coated with 500μl of 1% w/v gelatin, two wells with 500μl 0.1% w/v collagen (Sigma) and 2 wells with 300μl of 50 μgml^{-1} fibronectin. Second passage endothelial cells were trypsinized and a single cell suspension produced. Equal numbers of cells were seeded into each well in a total volume of 2ml. After overnight attachment 10μl of rbFGF was added to one of the wells coated with each of the substrates to a final concentration of 10pM. Four days after seeding the cells were trypsinized and counted using a Coulter counter. The experiment was carried out in triplicate.

Results

The results are shown in Table 4.1. Fibronectin was the best substrate for attachment and proliferation, second was collagen and last gelatin. Cells grown on each substrate showed a proliferative response to rbFGF (10pM) with gelatin showing the biggest increase.

4.2.4 Growth on Millicell culture well inserts

BREC were grown on culture well inserts to investigate whether the release of bFGF from these cells was from the abluminal or from the basal surface. To do this a confluent

monolayer of cells was required on the surface of the insert in order to form a cell barrier between the upper and the lower compartments.

BREC grew on Millicell-CM inserts coated with fibronectin however two problems were encountered. 1. A confluent monolayer was very difficult to achieve on the surface of the insert membrane with gaps appearing in the monolayer as it neared confluence. This made it impossible to separate upper and lower compartments. 2. Very small volumes of serum-free medium were needed in order to achieve the same medium to cell ratios as used when bFGF was detected by RIA in BREC conditioned medium (Section 6.2.1). Consequently the culture inserts rapidly dried out even in a humidified atmosphere.

These problems were not resolved and information was not obtained on the polarity of bFGF release from BREC.

Addition	Substrate		
	Gelatin	Collagen	Fibronectin
Control	1.1 ± 0.2	3.8 ± 1.3	5.0 ± 0.3
+bFGF(10pM)	2.9 ± 0.5	5.3 ± 0.8	8.6 ± 1.0
%age increase	166	41	72

Table 4.1 Effect of substrate on BREC proliferation. Counts expressed as x 10^4/well (n=3)

4.2.5 Cryopreservation of BREC

The best medium for cryopreservation was assessed. Vials of BREC which had been frozen in 10% v/v DMSO or 20% v/v glycerol in either neat PDS or DMEM + 10% PDS were thawed after 2 months in liquid nitrogen and seeded into 75cm^2 flasks, 1 vial per flask. The following day flasks were examined for cell attachment. Very few cells had attached to the substrate from those which had been frozen in either glycerol or neat PDS however most of the cells frozen in 10% DMSO in DMEM containing 10% PDS attached and proliferated.

After 5 days these cells were trypsinized and passaged after which they continued to grow well.

Several batches of BREC were routinely frozen in 10% DMSO in DMEM + 10% PDS. After 1 year in liquid nitrogen the cells were thawed and their viability assessed by Trypan blue exclusion. The results are shown in Table 4.2.

Date of freezing	Time in liquid N_2	Viability (%)
20/5//90	384 days	77
16/7/90	327 days	79
22/8/90	290 days	87
27/8/90	285 days	72
20/9/90	261 days	71

Table 4.2 The viability of frozen BREC

All batches showed similar viability and when cells were seeded in 25 cm^2 flasks greater than 50% of cells attached in each case and the cells grew well in subculture.

One batch of frozen BREC was trypsinized and seeded in 12 wells of a 24-well cell culture plate alongside 12 wells of recently isolated, non-frozen 1st passage BREC. 3 wells of both groups of cells were treated with 100μl of each of the following:- 1. DMEM + 0.1% serum (control); 2. DMEM + 10% serum (+ v.e.); 3. rbFGF (5pM); 4. rbFGF (20pM) and cell proliferation was measured by methyl-^3H thymidine incorporation. The results are shown in Fig. 4.7 expressed as the percentage increase in incorporation over the control.

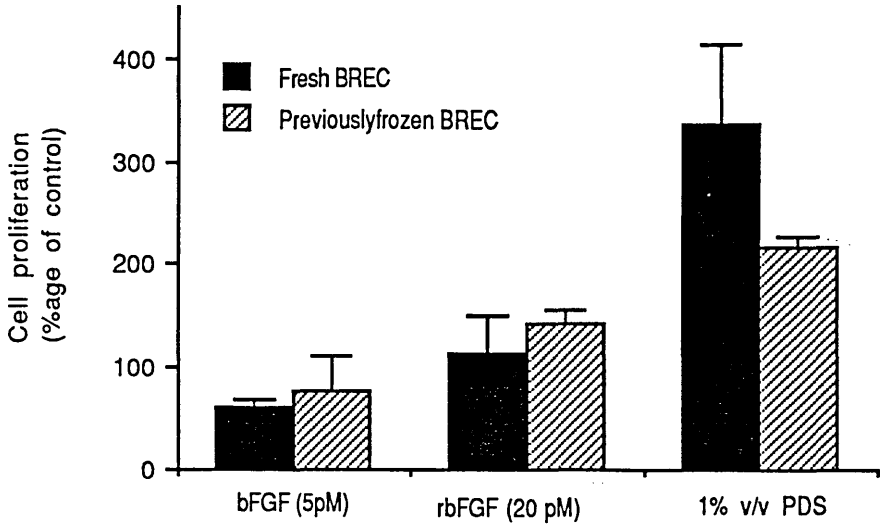


Fig. 4.7 Comparison of methyl-^{3}H thymidine incorporation into fresh and previously frozen BREC (mean ± SEM; n=3)

Frozen BREC responded to bFGF and an increased serum concentration with a similar proliferative response to that shown by fresh cells. The results therefore indicate that frozen batches of cells could be used in experiments where they were used to detect or examine such a proliferative response.

4.3 Human astrocytoma cells

Astrocytoma cells were stored frozen in liquid nitrogen as described for BREC above, until used. They had a high viability after thawing and grew well in subculture. There were two distinct cell morphologies in these cultures, a large rounded cell and a thin spiky cell (see Fig. 4.8)

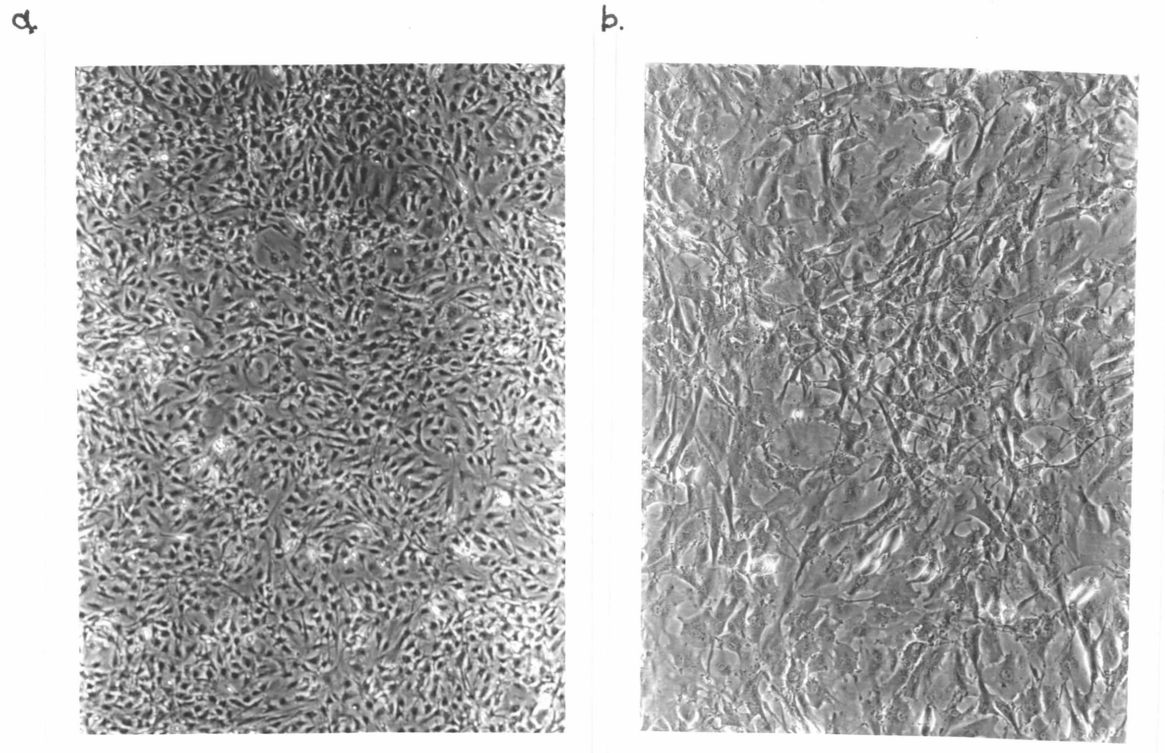


Fig. 4.8 Human astrocytoma cells in culture (a) x40 (b) x100

Upon reaching confluence the cells overgrew in a dense mat producing focal nodules of cells which were clearly visible to the naked eye.

4.4 Discussion

Two methods for the isolation and culture of BREC and BRP were assessed and the method of McIntosh et al., (1988) was preferred to that of Wong et al., (1987). The latter method allows the separation of capillaries from larger arterioles and venules and some studies, for example on capillary permeability, may require cells derived only from capillaries. However, in proliferative diabetic retinopathy new vessels arise not from the capillaries but from the venules and therefore it is as valid to use endothelial cells grown from fragments of the larger retinal vessels as it is to use cells derived from capillaries in experiments on the mechanisms of retinal angiogenesis.

BRP grew well using both methods of isolation but the vessel fragments adhered more reliably to the fibronectin substrate than to gelatin when BREC were being grown. Subcultured cells also adhered more readily to fibronectin

than to collagen or gelatin. BREC grew better on fibronectin than on the other substrates. However, their final density was dependent on their initial adherence to the substrate and thus a direct comparison could not be made. McIntosh et al., (1988) showed increased BREC growth of subcultured BREC on gelatin and collagen compared to fibronectin, although no difference was found by Schor & Schor, (1986). BREC grown on gelatin showed a greater percentage increase in cell proliferation in response to bFGF than when grown on collagen or fibronectin. This is similar to results showing an increased growth response of BREC to bFGF when the cells were cultured on gelatin as opposed to an extracellular matrix prepared from bovine corneal endothelial cells (Gospodarowicz et al., 1986b). We have however shown a 72% increase in the proliferation of BREC grown on fibronectin in response to bFGF indicating that cells grown on this substrate could be used in a bioassay for growth factors.

Both BREC and BRP showed typical morphologies in culture and were positively identified by immunofluOrescent staining for Factor VIII-related antigen and 3G5 ganglioside antigen respectively. The primary cultures were not however pure with BREC cultures contaminated with BRP and vice versa and the proportion of contaminating pericytes increased after the endothelial cells reached confluence. Other investigators have used pericyte weeding and cell cloning techniques to produce pure cultures of BREC (Bowman et al., 1982; Schor & Schor, 1986). We have found that the use of selective growth substrates, media and trypsinization substantially increased the purity of cultures following passage. BRP were visually homogenous after first passage, however BREC still contained up to 5% contaminating pericytes although this improved on further subculture. Most of the experiments were carried out on second passage cells and although cells could be passaged up to 13 times and retained staining for Factor VIII in the 6th subculture, their morphology changed and the continued maintenance of all their differentiated characteristics could not be guaranteed.

When BREC were visibly confluent they continued to proliferate at the same rate. Evidence for this was provided

by a continued increase in the optical density of Coomassie blue stained cultures. These results suggest that the cells did not become growth inhibited. It is possible that BREC would have become quiscent at higher densities however the monolayer was disrupted by cells rounding up and being released into the medium as the cell density increased (see Section 6.5.3).

BREC did not grow well in subculture if they were allowed to grow to confluence before trypsinization. This could be due to the increased number of pericytes in these confluent cultures being transferred to the subculture. BRP have been shown to inhibit bovine adrenal capillary endothelial cell growth upon contact by the activation of TGFß (Antonelli-Orlidge et al., 1989) however, this effect was not seen with BREC (Capatendes & Gerritsen, 1989). Physical damage is another possible reason for the poor subsequent growth of cells trypsinized after confluence. This damage could be produced during the mechanical dissagregation required to break clumps of cells produced by the association of cells with the extracellular matrix.

The ability to use cell freezing to provide BREC for simultaneous experiments on several different batches was shown by the successful growth in subculture of BREC frozen for periods greater than 1 year. Although cell viability was reduced it was still greater than 75% and the cells responded to the addition of rbFGF with a similar increase in DNA synthesis to that shown by freshly isolated cells.

The use of the Millicell culture well inserts was not possible in these studies due to sub-confluent growth of BREC. However there are several alternative well inserts now available with different substrate characteristics and their use should be investigated.

Chapter 5. THE MEASUREMENT OF BREC PROLIFERATION IN RESPONSE TO SERUM FROM DIABETIC PATIENTS, RETINAL EXTRACTS AND PURIFIED GROWTH FACTORS

5.1 Introduction

The measurement of the growth promoting activity of clinical samples, retinal extracts and purified growth factors was examined in order to evaluate the contribution of these growth factors to retinal endothelial cell proliferation which is a component of the angiogenic response in proliferative diabetic retinopathy.

There are several methods available for measuring cell growth and proliferation. The most direct method is cell counting however this is a laborious procedure for adherent cell lines. Counting cells with a Coulter counter is the most objective method but at least 20,000 cells per sample were required for reliable estimates and this precluded its use with cells grown in 24-well or 96-well cell culture plates. The formation of aggregates of cells with ECM following trypsinization made it difficult to reliably produce a single cell suspension required for cell counting . Therefore cell counting was not used other than to estimate cell number prior to seeding cells in multiwell plates for subsequent cell growth experiments.

We have used two methods of measuring cell growth and proliferation to investigate the effect of selected growth factors on BREC proliferation. The first method measured total cell protein by Coomassie blue-G250 staining and the second measured DNA synthesis by methyl-^{3}H thymidine incorporation. Both these methods have advantages and disadvantages which are considered in the discussion.

An important question in diabetic retinopathy research is whether a circulating factor is involved in the stimulus to retinal angiogenesis and a study is presented in Section 5.2.2 which seeks to address this question. Two circulating factors with a proposed involvement in proliferative diabetic retinopathy are growth hormone and IGF-1 (see Section 1.1.5 and the effect of these on BREC proliferation is assessed.

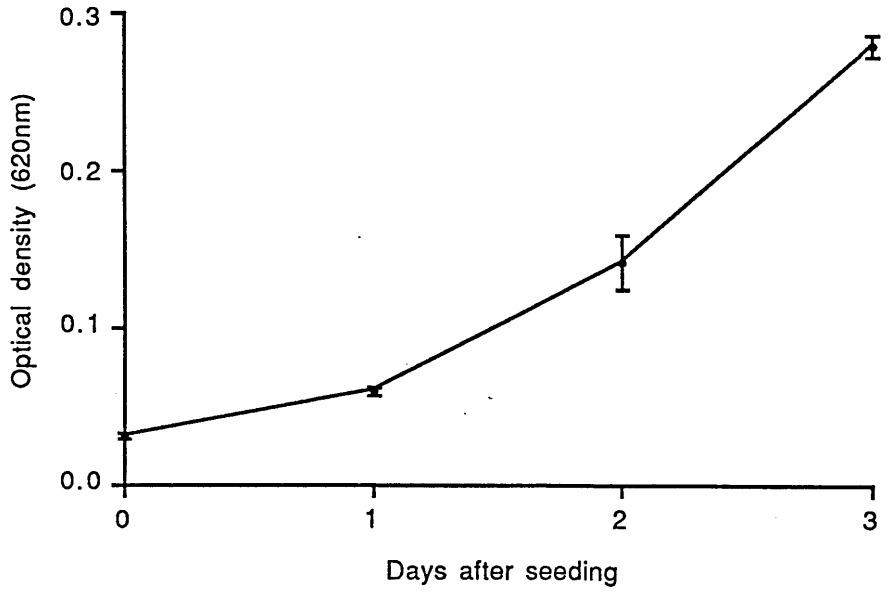
A comparison of Coomassie blue staining and methyl-^{3}H thymidine incorporation for the measurement of BREC proliferation in response to aFGF, bFGF and a bovine retinal extract was carried out.

5.2 BREC proliferation measured by Coomassie blue

5.2.1 Assay validation

The Coomassie blue assay was assessed for linearity of optical density against cell number and for linearity of proliferative response over the proposed time course of the assay. BREC were counted with a Coulter counter and seeded at a range of cell densities between 2000 and 10,000 cells per well in 96-well tissue culture plates. At 3 hours and 48 hours, cells were stained with Coomassie blue and the optical density determined. Alternatively cells were seeded at 4000 cells per well and 15 wells were stained and their optical density measured at 3, 24, 48 and 72 hours. The results are shown in Fig. 5.1.

Fig 5.1 (a)



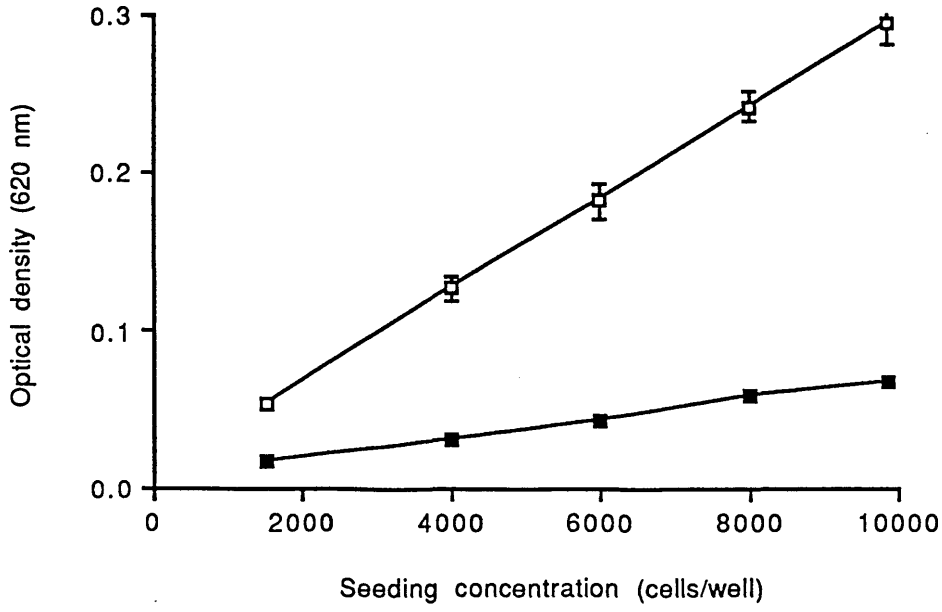


Fig 5.1 Optical density at 620nm (mean ± SEM; n=10) versus (a) time and (b) cell seeding concentration 3 hours (■) and 48 hours (□) after seeding

The results show that staining was directly proportional to cell number whether the cells were stained 3 or 48 hours after seeding.

The change in optical density with time shows an initial lag phase followed by a linear increase to 72 hours. The cells were visibly confluent at 60 hours although as already discussed, they continue to proliferate after reaching confluence. However, in order to prevent any possible effect of confluence on cell growth measurement in experiments, it was decided to seed the cells at 4000 per well and stain the cells at 48 hours, before confluence was reached.

In each experiment a different test substance or growth factor concentration was added to at least 10 wells of a 96-well culture plate. Because of possible plate to plate variation and differences in batches of cells, results were either compared within one plate or were related to a control sample on each plate. The coefficient of variation of the optical density at 620 nm between rows on a plate where the cells had been treated identically was calculated for 5

seperate batches of cells. All were below 5% (2.0-4.5%)indicating that differences between rows greater than this would be significant.

5.2.2 Comparison of the Coomassie blue staining and the methyl-^{3}H thymidine response to increased serum concentration

Cells were plated into two 96-well culture plates and grown in either 2% or 5% v/v PDS. The pre-optimized methyl-^{3}H thymidine incorporation assay (see Section 2.6.2) was carried out on one plate whilst the other was stained with Coomassie blue-G250 after 48hrs.

Methyl-^{3}H thymidine incorporation between 24 and 48 hours after seeding was 26,500 ± 3,800 cpm at 2% PDS and 31,300 ± 2,900 cpm at 5% PDS (both mean ± s.d.;n=20) an increase of 18% ($p<0.05$). The increase in Coomassie blue staining between 2 and 5% PDS was 29% ($p<0.05$).

5.2.3 The effect of human diabetic serum on bovine retinal endothelial cell proliferation

The study was set up to determine whether serum from diabetics with pre-proliferative or proliferative diabetic retinopathy stimulated increased retinal endothelial cell growth which would suggest an involvement for circulating factors in the development of diabetic retinal neovascularization.

Methods

Blood was collected from 27 diabetic patients attending the Diabetic Retinopathy clinic and 8 non-diabetic control subjects. Of the diabetic group, 6 had background retinopathy, 8 pre-proliferative, 6 active-proliferative and 7 treated, inactive-proliferative retinopathy as determined by the Hammersmith grading system (Oakley et al., 1967). One non-diabetic provided a reference sample against which all the other samples were compared. Serum was aliquoted and stored at -20°C until used.

Samples were tested for their ability to stimulate BREC growth in 96-well cell culture plates at a final concentration of 5% v/v per well. Each 96-well plate was used to compare 2 samples against the reference serum using 20 wells for each. After 48 hours the wells were stained with Coomassie blue and the optical density at 620nm determined.

Results

The results are shown in Fig. 5.2. The mean absorbance value for each sample showed a coefficient of variation of between 6% and 14% (n=20). Mean values for each patient group were compared with that of the control group using unpaired student t-tests. There was no significant difference between these groups ($p > 0.05$).

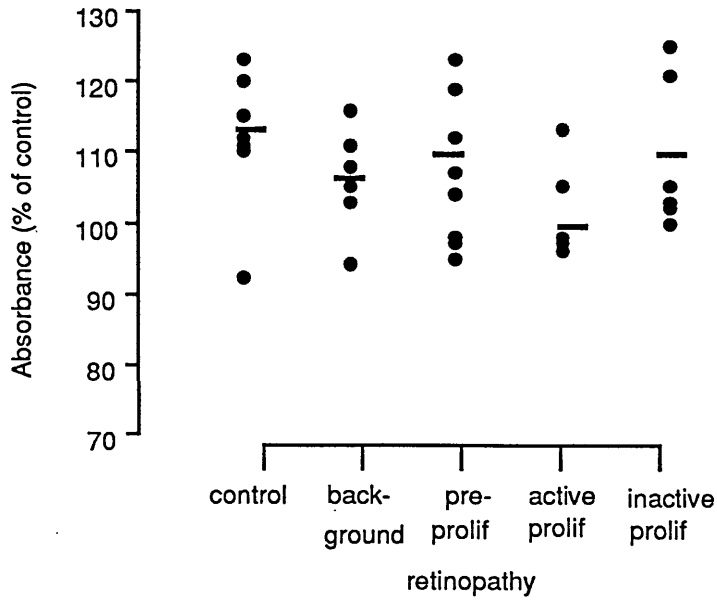


Fig 5.2 Mean absorbance value as a percentage of that for the reference serum for all samples subdivided by grade of retinopathy.

5.3 Effect of growth factors on BREC proliferation measured by Coomassie blue staining

5.3.1 Acidic and basic FGF

The dose response curves for a range of bFGF concentrations (0.6-10 pM) and a range of aFGF concentrations (6-610pM) is shown in Fig. 5.3. Both growth factors stimulated BREC growth with bFGF producing a maximum stimuation 40% greater than the control at 5pM and aFGF showing a maximum 55% increase in stimulation at 60pM The ED_{50}'s were 2.1pM and 14 pM respectively.

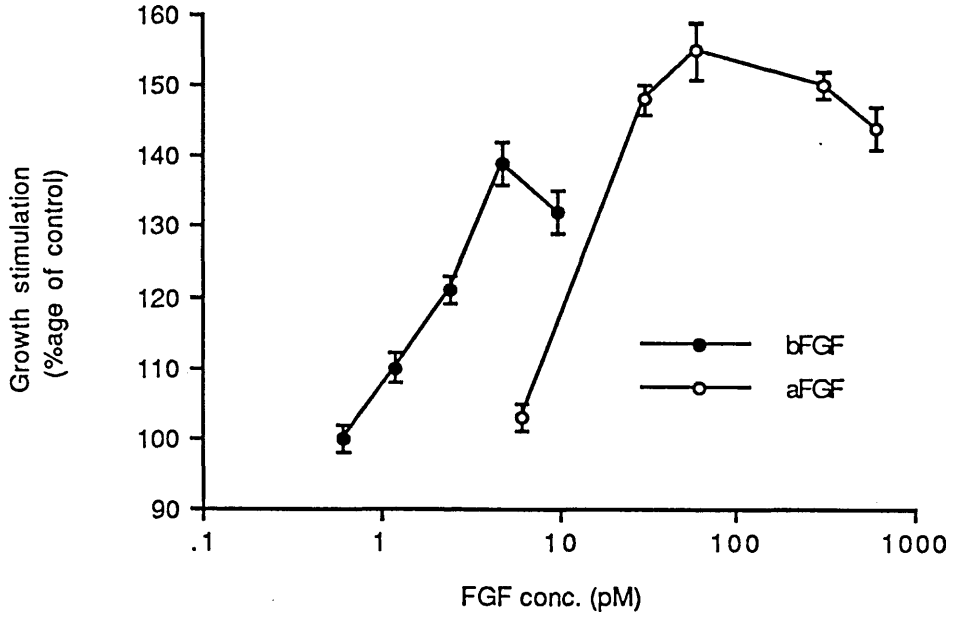


Fig. 5.3 Growth response curves for the stimulation of BREC by acidic and bFGF. Results are shown as mean optical density ± SEM expressed as a percentage of control versus growth factor concentration (n=10)

5.3.2 Retinal extract

Retinal extract was double diluted in sterile PBSA and 30μl of each dilution was added to each of 10 wells on two 96-well plates and growth stimulation was compared to that in

wells treated with 30 μl PBSA. The final PDS concentration was 7.5% v/v. The results in Fig. 5.4 show that retinal extract stimulated BREC proliferation 28% more than the control and this stimulation decreased with dilution until no difference was observed at 1:8 dilution.

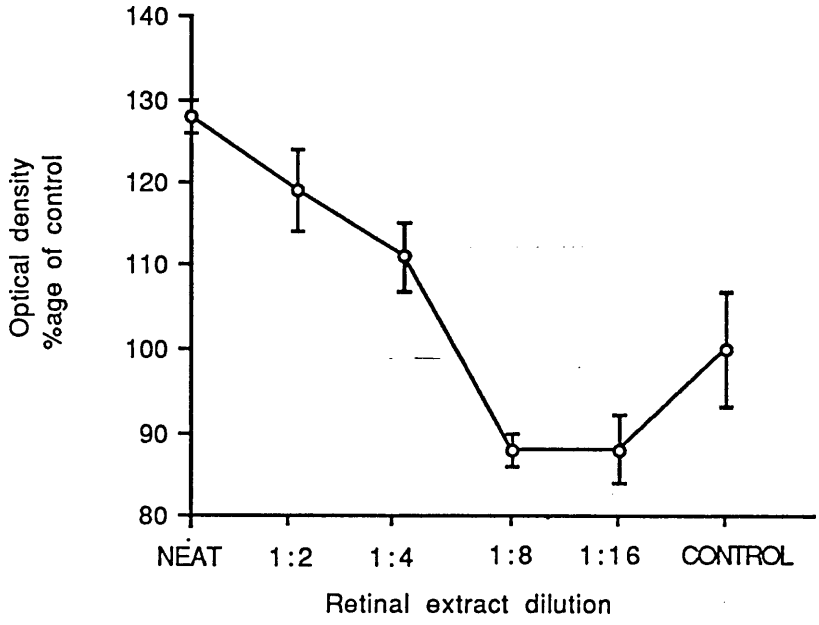


Fig. 5.4 Growth stimulation of BREC shown as mean optical density ± SEM expressed as a percentage of control for increasing dilutions of a retinal extract (n=10)

5.3.3 Bovine growth hormone

Bovine growth hormone, (obtained from the National Hormone and Pituitary Program of the National Institute of Diabetes, Digestive and Kidney Diseases, U.S.A.) was diluted to give a range of final concentrations (1-36nM) in 7.5% v/v PDS. Each concentration was added to 10 wells of a 96-well plate and the optical density at 620nM was measured after 48hrs following Coomassie blue staining.

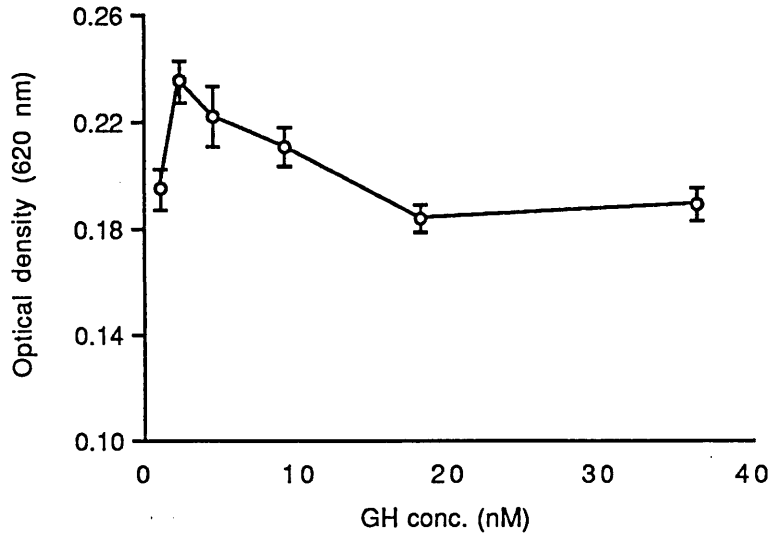


Fig 5.5 Optical density (mean ± s.d.; n=10) of BREC stained with Coomassie blue versus bovine GH concentration (nM).

Cell growth was greater at a growth hormone concentration of 2.3 nM compared with a concentration of 1.1 nM ($p<0.005$ using an unpaired Student t-test) and then declined as the concentration of growth hormone was increased further.

5.3.4 IGF-1

IGF-1 (a gift of Ciba-Geigy Pharmaceuticals, Horsham, U.K.) was diluted to provide a range of final concentrations from 8-128 nM in 7.5% v/v PDS. The growth stimulation at each concentration was measured in 10 wells compared to untreated control wells and was performed on two separate occasions. The range of optical densities after background subtraction was .026 to .078 and the coefficient of variation within concentration groups was below 16%. Results are shown in Fig. 5.6(a) A separate experiment was carried out with a range of final IGF-1 concentrations from 1.6 to 26 nM and the results expressed as mean o.d. ± SEM are shown in Fig. 5.6(b).

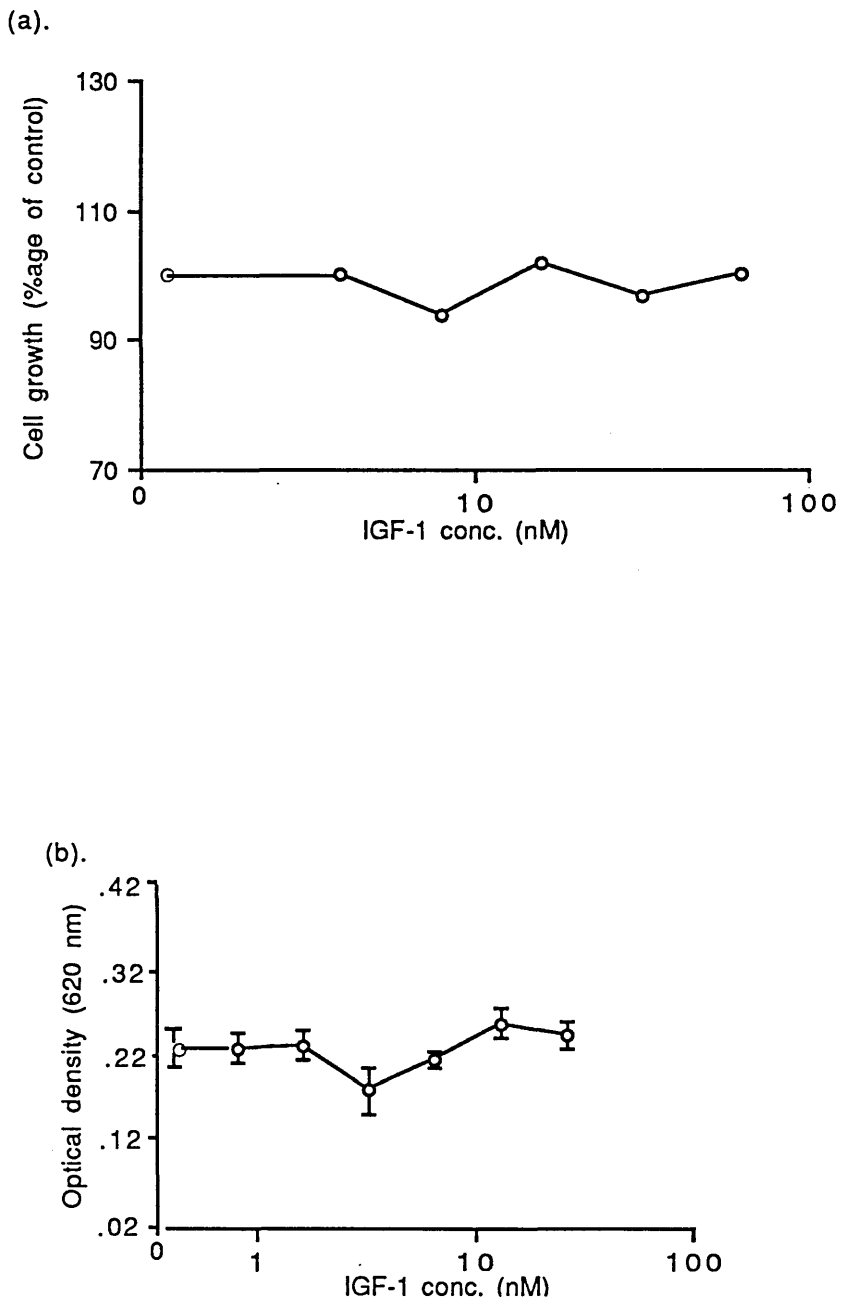


Fig 5.6 IGF-1 stimulation of BREC proliferation. Results are expressed as (a) a percentage of those in untreated wells, mean of two separate experiments against concentration and (b) optical density (620nm) versus concentration (n=10)

No significant stimulation of BREC growth was seen at any concentration of IGF-1.

5.4 Methyl-^{3}H thymidine incorporation assay of cell proliferation

Some of the results presented in this section use a methyl-^{3}H thymidine incorporation assay that was performed in 96-well tissue culture plates and had not been optimized for BREC seeding density, these will be indicated in the text. Most of the results use the optimized assay in 24-well tissue culture plates.

5.4.1 Assay optimization

A series of experiments was set up to optimize a number of variables in the thymidine incorporation assay. The variables were; cell seeding density, the serum concentration used in the pre-incubation, the serum concentration used during incubation of the cells with test substance and the length of time of this incubation. It had already been determined using the Coomassie blue assay that bFGF at a concentration of 9pM would stimulate BREC proliferation so the thymidine incorporation response at this concentration of bFGF was used as a measure of the sensitivity of the technique. All experiments were carried out in 24-well tissue culture plates. Results are expressed as cpm.

Cell seeding density

Cells were seeded at 2500, 5000, 10,000 and 20,000 cells per well and half the wells at each concentration were treated with rbFGF (9pM). Results are shown in Fig. 5.7 as the mean of duplicate wells from two separate plates.

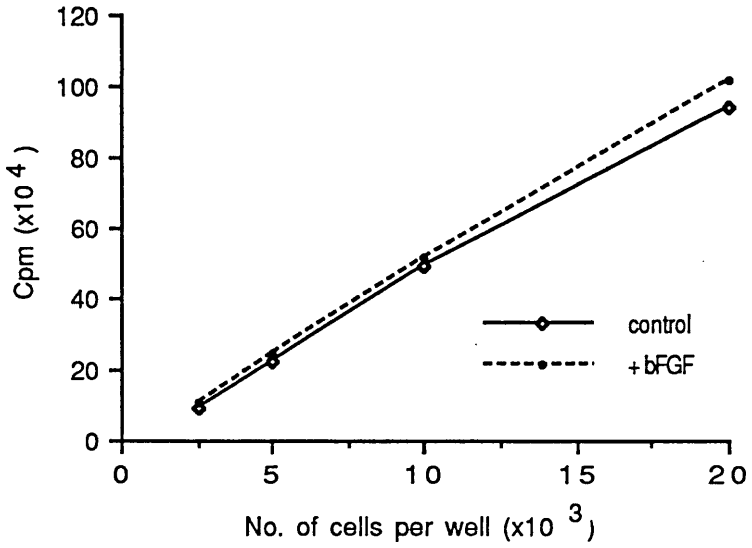


Fig. 5.7 The effect of cell seeding density on methyl-^{3}H thymidine incorporation and on the response of BREC to growth stimulation with rbFGF (mean ± SEM; n=4)

Methyl-^{3}H thymidine incorporation after 48hrs showed a linear increase with cell seeding concentration. Basic FGF (9pM) stimulated increased incorporation at all seeding densities. It was 20.8% greater at 2500 cells per well, 10.4% at 5000, 4.0% at 10,000 and 8.1% at 20,000. The graph of methyl-^{3}H thymidine incorporation against cell seeding density with added rbFGF was parallel to that with no rbFGF additions. It was decided to use a seeding concentration of 5000 cells per well for the experiments. Cells seeded at this density responded to bFGF with a significant increase in methyl-^{3}H thymidine incorporation and would presumably be capable of responding to other growth factors. They would also not grow to confluence over the time course of the experiment (see Section 5.2.1) and would not have the problem of poor cell growth seen at the lower cell density. Finally, 2.8% of the methyl-^{3}H thymidine added was incorporated at this cell density. This is sufficient to eliminate the effects of incorporation of trace impurities on the estimation of thymidine incorporation (Oldham, 1971).

Serum concentration and incubation time

Cells were seeded at 5000 per well in DMEM containing 0, 0.1, 1.0 or 10% v/v PDS. After 24 hours incubation 2 wells at each concentration were changed to either 0, 0.1, 1.0 or 10% v/v PDS containing methyl-^{3}H thymidine. Recombinant bFGF (9pM) was added to half of the wells at each concentration. After 24hrs the trichloroacetic acid precipitable material was harvested and thymidine incorporation measured. In a separate series of duplicate plates the above experiment was repeated with the exception that only 0 and 0.1% pre-incubation serum concentrations were used and incubations were carried out for 48hrs prior to harvesting.

The results are shown in Table 5.1 and are expressed as the percentage increase in thymidine incorporation in the rbFGF containing wells compared to control wells at each combination of serum concentrations.

Pre-incubation serum conc.(%)	Serum conc. (%) during incubation			
	10	1	0.1	SF
10	5.5	10.4	-0.9	0.1
1	-5.7	-2.6	5.2	10.9
0.1	4.5	-2.0	7.6	15.2
SF	0.9	13.4	6.1	6.1

Pre-incubation serum conc. (%)	Serum conc. (%) during incubation			
	10	1	0.1	SF
0.1	7.9	4.3	12.5	-4.3
SF	15.9	6.1	5.4	32

Table 5.1 Percentage increase in methyl-^{3}H thymidine incorporation in response to rbFGF (9pM) at a range of pre-incubation and incubation serum concentrations with (a) a 24hr incubation and (b) a 48hr incubation

The growth response to rbFGF after an incubation time of 24hrs was variable with decreased methyl-^{3}H thymidine incorporation at some pre-incubation and incubation serum concentrations. Pre-incubation with serum-free medium produced consistent stimulation with rbFGF at all incubation serum concentrations. However, there was significant cell death during the pre-incubation stage in these wells which could contribute to error in the measurements. The requirement for a pre-incubation stage was to starve the cells of serum and increase their sensitivity to added growth factors. An increase in the serum concentration from the pre-incubation to the incubation stage stimulated cell growth although this did not affect the response to rbFGF. It was decided to use an 0.1% serum concentration for the pre-incubation stage and 0.1% for the incubation stage. At these serum concentrations, increasing the length of the incubation stage to 48hrs increased the amount of methyl-^{3}H thymidine incorporated by 74% in the control wells and 181% in the rbFGF treated wells. This increased counting precision but did not diminish the growth response of the cells to rbFGF. Forty eight hours was therefore chosen as the length of the incubation stage.

Effect of harvesting method on methyl-^{3}H thymidine incorporation

The standard method was as follows; the cells were washed twice with cold PBSA, insoluble material was precipitated with one 5 min incubation with ice cold 10% w/v TCA followed by a further 5 min incubation with ice cold 5% w/v TCA. Water was removed by adding 100% v/v cold ethanol prior to solubilizing the cells in 25M formic acid and counting ^{3}H-thymidine incorporation in scintillation fluid. It was noted that material was frequently lost during this procedure. This led to poor duplication and this was attributed to the 10% TCA wash. An experiment was set up to examine the influence of the harvesting steps on thymidine incorporation.

Cells were seeded at 5000 per well in a 24-well plate and after pre-incubation in 0.1% v/v PDS half the wells were

incubated in 0.1% PDS and half in 1% PDS each containing methyl-^{3}H thymidine. Two wells in each treatment group were harvested using either the standard method or omitting the 5% TCA wash, the 10% TCA wash, both washes or solubilising the cells after PBSA washes only. The cpm measured as the mean in each pair of wells is shown in table 5.2

Serum(%)	10% / 5% TCA	10% TCA	5% TCA	Ethanol	None
0.1	8861	8761	8355	9968	9361
1	11738	12019	11469	11740	11777
%age increase	132 —	137	137	118	126

Table 5.2 Effect of the harvesting method on methyl-^{3}H thymidine incorporation expressed as cpm.

The results showed that precipitation with ice cold 5% w/v TCA was sufficient to remove most of the soluble ^{3}H-thymidine. This precluded the need for a wash with 10% w/v TCA which could wash away cell material.

Experimental design

The optimised methyl-^{3}H thymidine incorporation assay was used to investigate the effects of added growth factors on DNA synthesis by BREC. Each concentration of the growth factor used was added to triplicate wells of a 24-well plate alongside negative controls containing either 0.1% v/v PDS or no serum (SF) and positive controls containing 1% v/v PDS. The positive controls were used to ensure that each batch of cells could respond to added serum.

For the results of some of the assays, methyl-^{3}H thymidine incorporation is expressed as a percentage of incorporation in negative control wells. The coefficient of variation between triplicate wells was assessed using 4 x 24-well tissue culture plates seeded with BREC and was

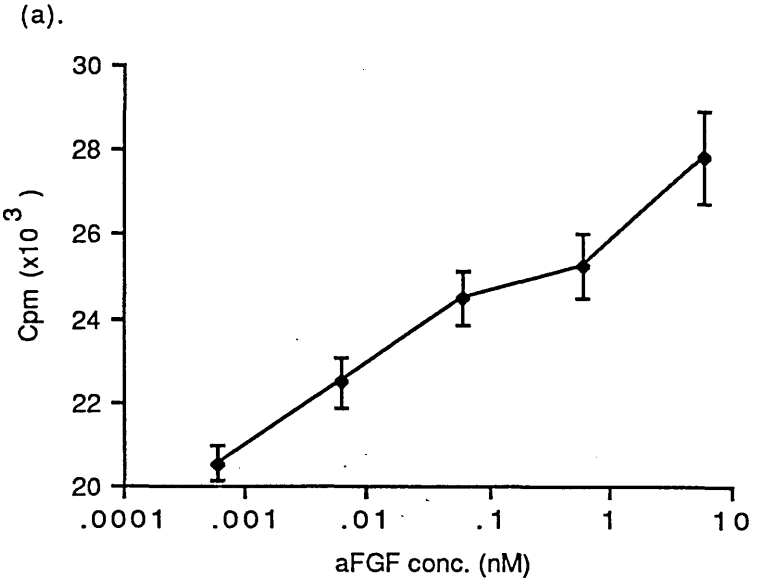
calculated as 15.2%. Therefore, differences between triplicates greater than this could be considered significant.

5.5 Effect of growth factors on BREC and BRP proliferation measured by methyl-^{3}H thymidine incorporation

5.5.1 Acidic and basic FGF

Dose response curves for the stimulation of methyl-^{3}H thymidine incorporation into BREC by aFGF were produced before the assay optimization described in Section 5.4.1. Whereas those for bFGF were produced after assay optimization. The results for both are shown in Fig. 5.8 (a) and (b) with those for aFGF from one experiment (range 6-6000pM) and those for bFGF the mean ± SEM of three separate experiments (range 0.25-50pM).

Fig. 5.8 (a)



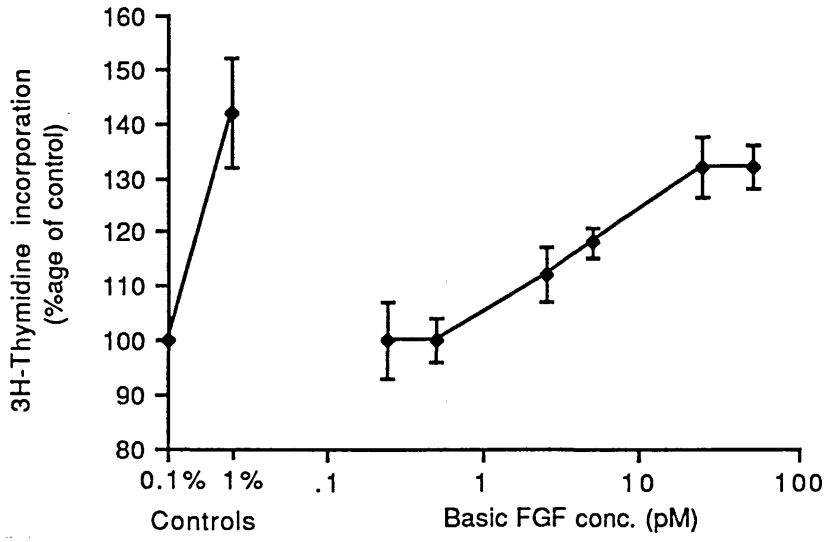


Fig. 5.8 The proliferative response measured by methyl-^{3}H thymidine incorporation of BREC to (a) aFGF (cpm x 10^3; mean ± SEM;n=10) and (b) bFGF expressed as % of control, mean ± SEM from 3 separate experiments) with the response of cells to 1% v/v PDS provided for comparison.

The results show that both a and bFGF stimulate BREC proliferation with bFGF being more potent. The ED_{50} value for aFGF was 27pM and that for bFGF 2.8pM.

The bioassay was to be used for measurement of the bioactivity of BREC conditioned medium which contained 0.01% w/v CHAPS. The influence of 0.01% CHAPS on the bFGF standard curve was carried out and the results are shown in Fig. 5.9

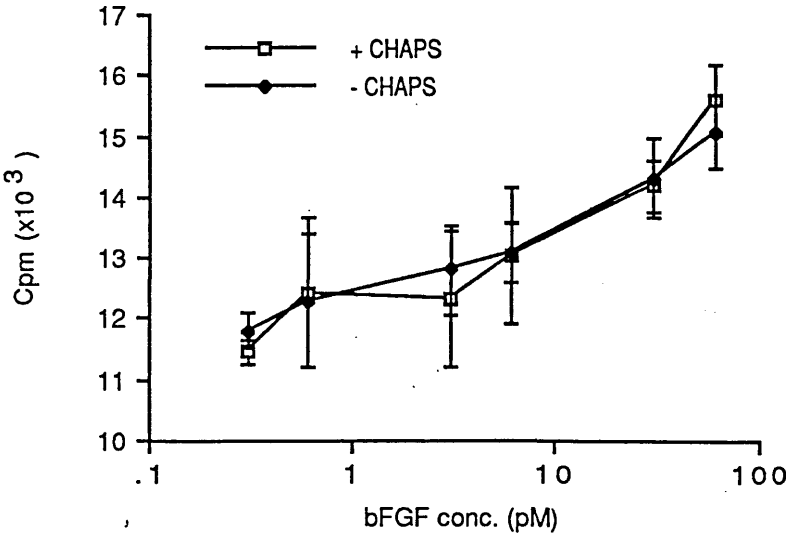


Fig. 5.9 The effect of 0.01% CHAPS on bFGF stimulation of BREC proliferation (mean ± SEM; n=3)

No effect of CHAPS on the proliferative response could be detected.

5.5.2 Retinal extracts

The influence of retinal extract on the proliferation of BREC was carried out before the optimization of the thymidine incorporation assay. 10 $\mu l ml^{-1}$ of retinal extract was added to 6 wells of two 24-well plates and thymidine incorporation compared to wells with 10μl PBSA alone added. This was carried out on two separate batches of cells. The results, expressed as mean cpm ± s.d (x 10^4); n=12, are for the first batch, control 2.7 ± 0.4 versus retinal extract 4.3 ± 0.7 an increase of 59% and for the second batch 7.5 ± 1.1, control versus 10.7 ± 2.3, retinal extract, an increase of 43%.

5.5.3 Growth hormone

The effect of a range of bovine growth hormone concentrations (0.05-10 nM) on BREC proliferation is shown in Fig. 5.10. The 1% v/v control serum increased methyl-^{3}H thymidine incorporation by 59.5% but no significant increasse was seen at any concentration of growth hormone.

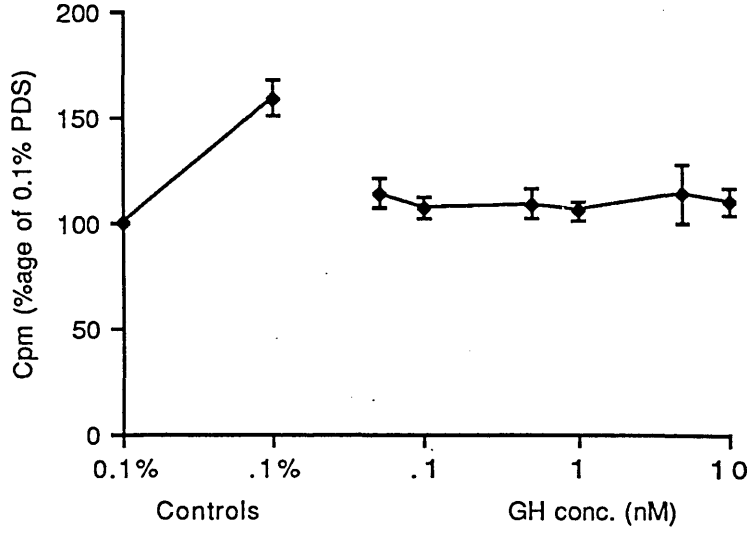


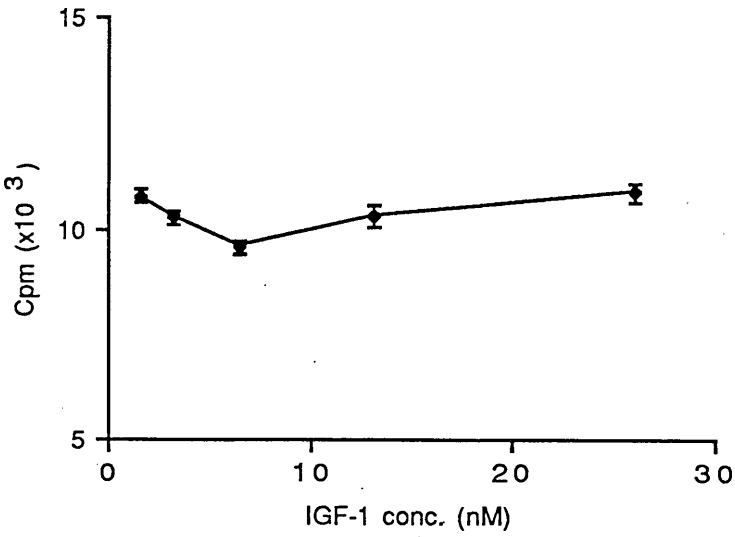
Fig. 5.10 The effect of bovine GH on BREC proliferation. Each point is the mean ± s.d. of data from 6 separate batches of cells. The effect of 1% PDS is shown for comparison

5.5.4 IGF-1

The effect of IGF-1 on the stimulation of BREC proliferation was carried out using thymidine assays both before and after optimization. The results are shown in Fig. 5.11 with graphs showing the mean ± SEM for 20 wells (pre-optimization) and 6 wells (after optimization) of data from 2 separate plates in each experiment. In the preoptimization experiment the incubations were carried out in serum-free DMEM containing 0.25% w/v BSA whilst the optimized assay used 0.1% v/v PDS.

There was no significant stimulation of BREC proliferation at any of the IGF-1 concentrations used.

Fig. 5.11 (a)



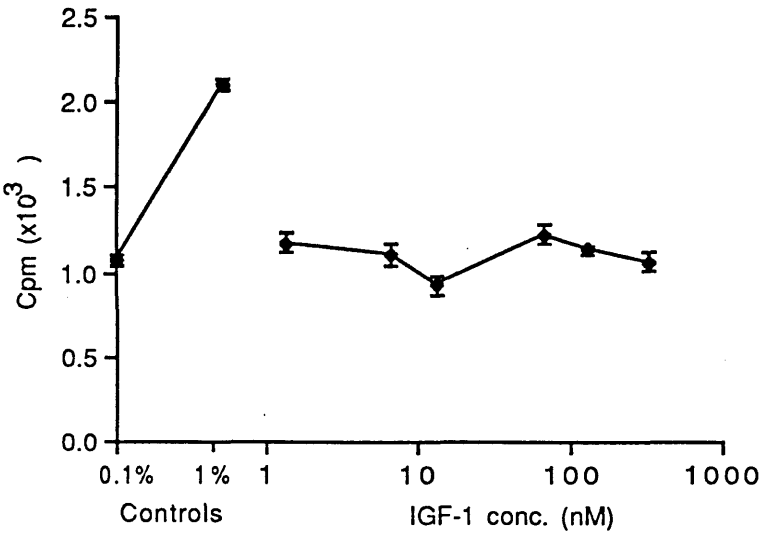


Fig. 5.11 Methyl-^{3}H thymidine incorporation in BREC in response to IGF-1. (a) preoptimized assay (mean ± SEM; n=20), (b) optimized assay (mean ± SEM;n=6). The effect of 1% PDS is shown for comparison

5.6 Interaction between bFGF and IGF-1

Although IGF-1 did not directly stimulate BREC proliferation it could act by interaction with other growth factors. Therefore its effect on the growth response to rbFGF was tested. The concentration of IGF-1 used was 1nM because this dose stimulated half maximal proliferation in BRP (Dr. M Porta, personal communication). It was also the minimum dose shown by King et al., (1985) to stimulate BREC proliferation. Dose response curves to rbFGF were carried out with and without 1nM IGF-1 in both serum-free DMEM and medium containing 0.1% v/v PDS. Both serum-free and serum-containing medium were used to assess the possible interference of IGF-1 binding proteins, present in the serum, upon IGF-1/bFGF interactions. The results are shown in Fig. 5.12.

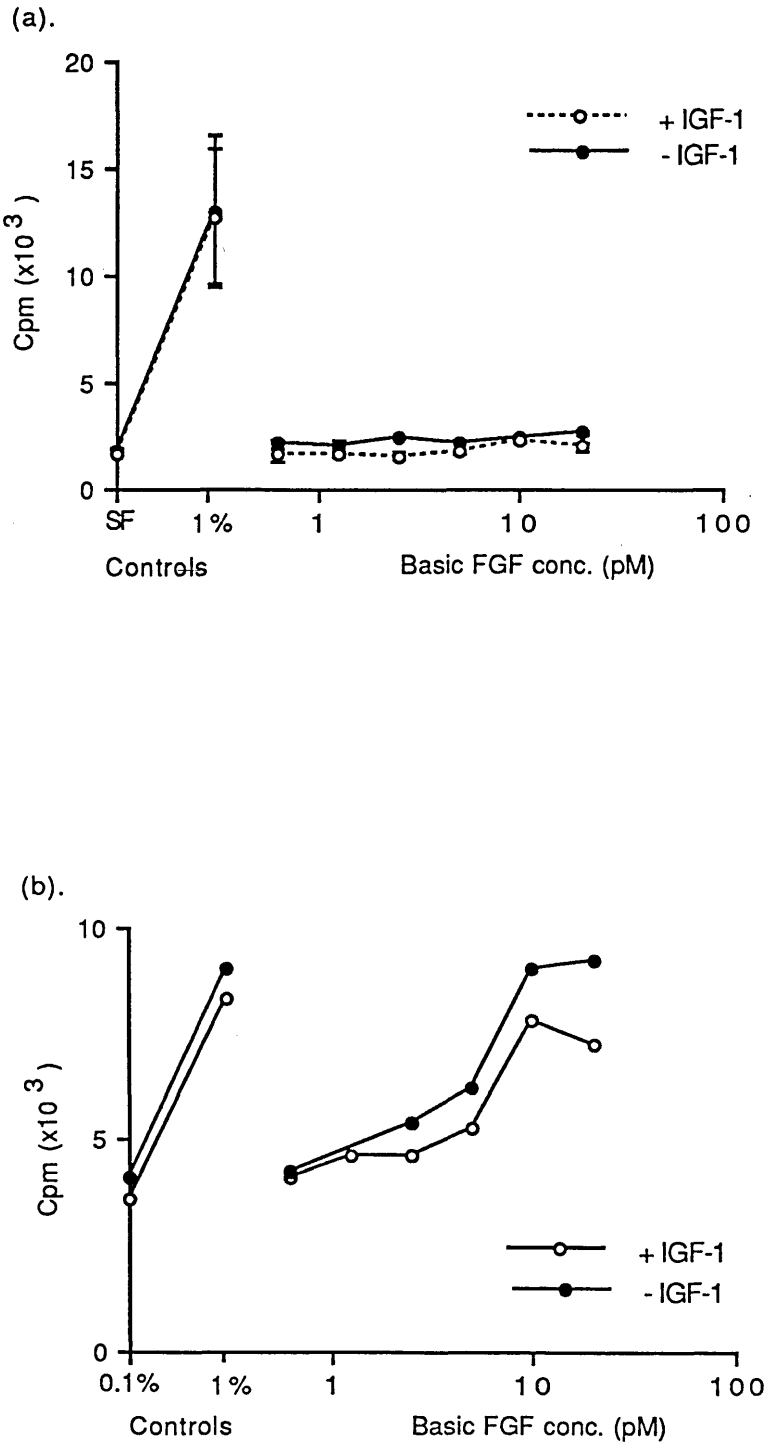


Fig. 5.12 The effect of IGF-1 on BREC growth response curves to bFGF assessed using methyl-^{3}H thymidine incorporation in (a) serum-free medium (mean ± s.d. from 3 batches of cells) and (b) medium containing 0.1% v/v PDS (mean of triplicate wells)

The results show no additive or synergistic effect of IGF-1 on the growth response of BREC to rbFGF. In serum-free medium the response appears to be attenuated but because of the low methyl-^{3}H thymidine incorporation and large variability in the response to rbFGF at different concentrations under these conditions it is difficult to interpret the data.

5.7 Discussion

The results of the optimisation of the Coomassie blue assay showed that the optical density of stained BREC increased with time in culture and was directly proportional to the original cell number when cells were stained at 3hrs or 48 hrs. The use of Coomassie blue to measure protein concentration has been criticised on the grounds that there is not a linear relationship between these two parameters. (Splittgerber and Sohl, 1989). There is however a linear relationship between staining and cell number so this technique is not affected. A second criticism is that Coomassie blue staining does not necessarily reflect cell proliferation and this is more difficult to refute. Cells can grow in size as well as increase in number and protein production could vary between treatments. There was no visual evidence for a change in cell size in our cultures provided the cells were stained at 48hrs, before they became over-confluent. It is not however certain whether small changes in cell size or differences in protein synthesis would influence the optical density and lead to false interpretation of results. For this reason the methyl-^{3}H thymidine assay was developed. The Coomassie blue assay had the advantages of being more straightforward, could be carried out in 96-well plates using small numbers of cells, provided visual evidence of wells where cells had been washed away so that results from these wells could be disregarded and was more sensitive than the methyl-^{3}H thymidine incorporation assay. The increased sensitivity was due to a coefficient of variation between treatment groups of less than 5% compared to a

coefficient of variation of 15.2 % between the means of triplicate wells in the methyl-^{3}H thymidine incorporation assay. Modifications to the Coomassie blue assay have been introduced (McIntosh et al., 1988) providing further increases in sensitivity.

The methyl-^{3}H thymidine incorporation assay on BREC proved to be difficult to establish in practice. The main problem was that the cells did not become quiescent and the assay had to be carried out on growing cultures. There was therefore high basal thymidine incorporation. In an attempt to reduce this and produce growth arrested cultures so that pulse labelling could be used, the cells were serum starved for 48hrs in 0.5% v/v PDS and 0.25% w/v BSA as described by King et al., (1985). The cells however continued to proliferate and after they became confluent, many cells rounded up and were lost from the monolayer into the medium so no reliable results were obtained. Starving the cells of serum for 24hrs in 0.1% v/v PDS at a low cell seeding density was introduced to reduce basal thymidine incorporation and make the cells more sensitive to added factors whilst preventing cell death. This method did not however produce the growth arrest and consequent cell synchronisation required for pulse labelling studies and methyl-^{3}H thymidine incorporation was therefore carried out over 24 or 48 hours. The use of 0.1% PDS for both the pre-incubation and incubation stages produced the most reliable response to bFGF.

Although the methyl-^{3}H thymidine incorporation assay does not have the same problems as Coomassie blue staining it does have significant drawbacks. False results may be generated due to the effects of test agents on thymidine uptake or to catabolism of the labelled precursor and possible incorporation of the degraded products into acid insoluble macromolecules (Oldham, 1971). More fundamentally, methyl-^{3}H thymidine incorporation measures DNA synthesis and not cell division and although these processes often go together in the cell cycle they can also be separated (Baserga, 1989).

The difficulties posed by counting cells from large numbers of replicate wells in many experiments means that

other methods have to be used. However, because of the drawbacks of the two techniques discussed above for measuring cell proliferation it is advantageous if results using one technique can be confirmed by another.

In the study on the effect of human diabetic serum on the proliferation of BREC we showed no increase in the growth of these cells when exposed to sera from patients with background, pre-proliferative, proliferative or inactive (treated) proliferative retinopathy. This is in contrast to the results of Petty et al., (1988) who showed that serum from patients with retinal neovascularisation stimulated ^{3}H-thymidine incorporation into human umbilical vein and omental endothelial cells significantly more than serum from patients with background retinopathy. Methodology differences between the techniques used for measuring cell proliferation are unlikely to be responsible for the discrepancy in these results. Coomassie blue staining was more sensitive at measuring growth stimulation to increased serum concentration than methyl-^{3}H thymidine incorporation (29% vs. 18% increase). It is possible that differences in cell type are responsible for our inability to show increased cell growth in response to serum from diabetics with proliferative diabetic retinopathy. Retinal endothelial cells differ both morphologically and metabolically from endothelial cells found in other vascular beds (Zetter, 1981; Wong et al., 1987). However, species differences are likely to be more important. In support of this, 5 sera from patients with proliferative diabetic retinopathy used in our study, showed a 205% ± 27% (mean ± SEM) increase in ^{3}H-thymidine incorporation when compared to control sera using human umbilical vein endothelial cells but no increase was demonstrated in bovine aortic endothelial cells. (R Petty, personal communication). The stimulatory factor for human umbilical vein endothelial cells in sera from patients with proliferative diabetic retinopathy is unlikely to be bFGF because this growth factor was able to stimulate BREC proliferation. These results confirm the absence of measurable bFGF in diabetic sera seen using immunoassay.

Both aFGF and rbFGF stimulated BREC proliferation as

measured by both Coomassie blue staining and methyl-^{3}H thymidine incorporation with rbFGF being more potent. This is in agreement with the results of other investigators (Gospodarowicz et al., 1986b; Bensaid et al., 1989a). The ED_{50} values for rbFGF were 2.1pM measured by Coomassie blue staining and 2.8pM measured by methyl-^{3}H thymidine incorporation. This compares with values for the stimulation of BREC proliferation by purified pituitary bFGF of 0.8pM (Gospodarowicz et al., 1986b) and 5.5pM (Bensaid et al., 1989a) measured by cell counting. The ED_{50} values for aFGF measured by us using Coomassie blue staining and methyl-^{3}H thymidine incorporation were 14pM and 27pM respectively. This agrees with the data of other investigators showing that aFGF is a less potent mitogen than bFGF (Lobb & Fett, 1984; Courty et al., 1985). However, the ED_{50} values obtained by Gospodarowicz et al., (1986b) and Bensaid et al., (1989a) were 161pm and 193pM respectively which were considerably higher than our results. Thomas et al., (1985) showed an ED_{50} of 15pM for the stimulation of ^{3}H-thymidine incorporation into mouse lung capillary endothelial cells by aFGF, with a similar activity on human vascular endothelial cells and the potency of this purified growth factor may be dependent on the batch used. Our data confirms the potent effects of bFGF on the stimulation of BREC proliferation which are 20- to 60-fold lower than those seen for the stimulation of DNA synthesis by TGFß, EGF and PDGF in responsive non-transformed cell types (Gospodarowicz et al. 1987a).

Retinal extracts stimulated the proliferation of BREC when measured by either Coomassie blue staining or methyl-^{3}H thymidine incorporation. Retinal extracts were shown to stimulate BREC proliferation by previous investigators (Gitlin & D'Amore, 1983; Wong et al., 1987) whilst McIntosh et al., (1989) demonstrated that a combination of crude retinal extract and PDS inhibited BREC growth. Our measurements of retinal extract activity using the Coomassie blue assay were carried out in medium containing 7.5% v/v PDS however, the inhibitory activity shown by McIntosh et al., (1989) only became evident after 3 days and in our assay cells were exposed to the retinal extract for 48 hours only.

Both basic and aFGF are present in these extracts (see Section 6.4) and are probably major contributors to its stimulatory activity although it is possible that other growth factors, for example, ESAF are also present (Kissun et al., 1982).

The results with bovine growth hormone are more equivocal than those for the fibroblast growth factors. There was an increase in cell growth as measured by Coomassie blue staining at growth hormone concentrations of between 1.1nM and 2.3nM but this increase was not sustained at higher growth hormone concentrations. There was however no increase in BREC proliferation over the same concentration range when measured by methyl-^{3}H thymidine incorporation. A possible explanation for the lack of response of BREC to growth hormone when measured by methyl-^{3}H thymidine incorporation is a requirement for serum. Rymaszewski et al., (1991) showed an increased proliferation of human retinal endothelial cells in response to human growth hormone over the range 0.02-10nM measured using cell counting, ^{3}H-thymidine incorporation and increased DNA content however this stimulation of cell proliferation was only seen when growth hormone was added with at least 2.5% v/v serum. Bovine growth hormone added at concentrations of 0.125nM and 10nM in serum-free medium had no effect on BREC proliferation (King et al., 1985). Growth hormone present in human serum is not likely to contribute to the proliferative effect of added hormone on BREC because of its species specificity (Wallis et al., 1985). Whether the observed stimulation of retinal endothelial cells by growth hormone is directly through growth hormone receptors or secondary to the stimulation of IGF-1 production is not known. However, as discussed below, we showed no effect of IGF-1 on BREC proliferation.

Plasma growth hormone concentrations fluctuate markedley over 24 hours however, although there is conflicting data, it is generally agreed that GH concentrations are higher in type I diabetic patients compared to non-diabetic controls (Hansen, 1972; Hayford et al., 1980). The mean integrated GH concentration of 48 half-hourly measurements was significantly higher in 46 type I diabetic patients (0.41 ±

0.26 nM; mean ± s.d) compared to age and sex matched controls (0.18 ± 0.05 nM; $p<0.001$) (Hayford et al., 1980). These values are lower than those shown by us to stimulate BREC proliferation however the levels producing increased proliferation in human retinal endothelial cells (0.02-10nM) are in the range at which the increased GH secretion seen in diabetes could be of relevance. The contribution of circulating GH to endothelial cell proliferation during retinal angiogenesis has still to be clarified.

IGF-1 did not stimulate BREC proliferation when measured using either Coomassie blue staining or methyl-^{3}H thymidine incorporation. These results differ from those of King et al., (1985) who showed a dose related increase in ^{3}H-thymidine incorporation into BREC over the concentration range 1-100nM. Stimulation of capillary endothelial cell proliferation by IGF-1 (1-100nM) has also been shown by Bar et al., (1988). Differences in the assay method could be responsible for our contrasting results. We have tried using the same method as that of King et al., (1985) but did not obtain reliable results (see above). Another possible reason for the discrepancy is that BREC were cultured on different substrates. King et al., (1985) used gelatin and although we have shown that BREC grown on gelatin, collagen and fibronectin respond to bFGF with increased proliferation this may not be true for IGF-1.

IGF-1 did not have any effect on the proliferative response of BREC to bFGF. However FGF and IGF-1 have been sown to act synergistically to stimulate DNA synthesis in rat aortic smooth muscle cells probably via an increase in the number of IGF-1 binding sites (Pfeifle et al., 1987). A difference in the cell type used could be responsible for our different results. Our data does not accord with the idea that bFGF is a competence factor which requires IGF-1 as a progression factor in order to maximally stimulate cell proliferation. However we found no response of BREC to IGF-1 and it is possible that IGF-1 is not a progression factor for these cells.

Chapter 6. CHARACTERIZATION OF bFGF PRODUCTION BY BREC

6.1 Introduction

Basic FGF has been extracted from a wide range of tissues and cells (see Section 1.3.5) but early studies on cultured endothelial cells suggested that bFGF remained cell associated, either in the ECM or within the cell and was not secreted into the culture medium (Vlodavsky et al., 1987a; 1987b). These results were in agreement with the sequence of the cDNA clone which did not show a region coding for a classical signal peptide which allows packaging of the protein into secretory vesicles. At about the same time it was reported that bFGF could be detected in the culture medium of bovine adrenal cortex endothelial cells (Schweigerer et al., 1987a). These authors suggested that cell leakage or lysis could be responsible for the release of bFGF into the culture medium. They also showed that cell proliferation was reduced in the presence of anti-bFGF neutralizing antibodies indicating that bFGF can act as an autocrine stimulator of endothelial cell proliferation and these results have since been confirmed (D'Amore et al., 1990). Basic FGF is present in the ECM secreted by cultured endothelial cells (see Section 1.3.6) bound to heparan sulphate proteoglycans although how it leaves the cell and becomes associated with the matrix is still not clear.

The release of bFGF from retinal cells or from ECM is required if it is to be involved in retinal angiogenesis. It was therefore important to measure bFGF production, localization and release from BREC. We have used the RIA to measure bFGF in BREC conditioned medium, cell extracts and ECM and investigated the presence of acidic and bFGF in cell conditioned medium by immunoblotting. We have also used the ELISA in an attempt to increase the sensitivity of bFGF measurement.

The lack of a hydrophobic signal peptide precludes the secretion of bFGF by conventional mechanisms (Abraham et al., 1986a; Rifkin & Moscatelli, 1989) and therfore this is not a control point for its release. However, the amount of bFGF

available for release from damaged cells or for release by unknown mechanisms and deposition into the ECM could be regulated at the level of new protein synthesis. Basic FGF mRNA levels are very low in most tissues, however transcripts can be readily detected in a variety of normal and transformed cells in culture (Sternfeld et al., 1988). We have investigated the presence of bFGF mRNA transcripts in BREC. We have also looked at their regulation by serum, 12-O-tetradecanoylphorbol 13-acetate (TPA), a phorbol ester known to stimulate bFGF mRNA expression in human umbilical vein endothelial cells (Bikflavi et al., 1990) and cycloheximide, a protein synthesis inhibitor reported to stabilize bFGF mRNA transcripts in a glioblastoma cell line (Murphy et al., 1990).

6.2 Measurement by radioimmunoassay of bFGF in BREC conditioned medium, and cell and ECM extracts.

6.2.1 BREC conditioned medium

Bovine retinal endothelial cell conditioned medium was prepared from confluent flasks of BREC (see Section 2.5.2) as follows. Growth medium was removed, the cells washed with HBSS and serum-free DMEM containing 0.01% CHAPS was added (2ml/25cm^{2}flask or 5ml/75cm^{2}flask). The flasks were incubated at 37°C for 48hrs after which the medium was removed, centrifuged at 925g and the supernatant frozen at -20°C prior to assay.

Many batches were prepared and assayed in several RIA's over the course of the study. The amount of bFGF in BREC conditioned medium was 0.65 ± 0.27 pmol/10^{6}cells (mean ± s.d.; n=26; range 0.41-1.45 pmol/10^6 cells). The identity of bFGF immunoreactivity in BREC conditioned medium was checked by heparin-Sepharose chromatography (see below), immunoblotting (Section 3.4) and by testing for the removal of bFGF immunoreactivity after passage of BREC conditioned medium over a heparin-Sepharose column. The concentration of bFGF in BREC conditioned medium after one passage over a heparin-Sepharose column was reduced from 221pM to 62pM.

6.2.2 Heparin-Sepharose profiles of BREC conditioned medium

The method is in Section 2.2.5. and the results are shown in Fig. 6.1.

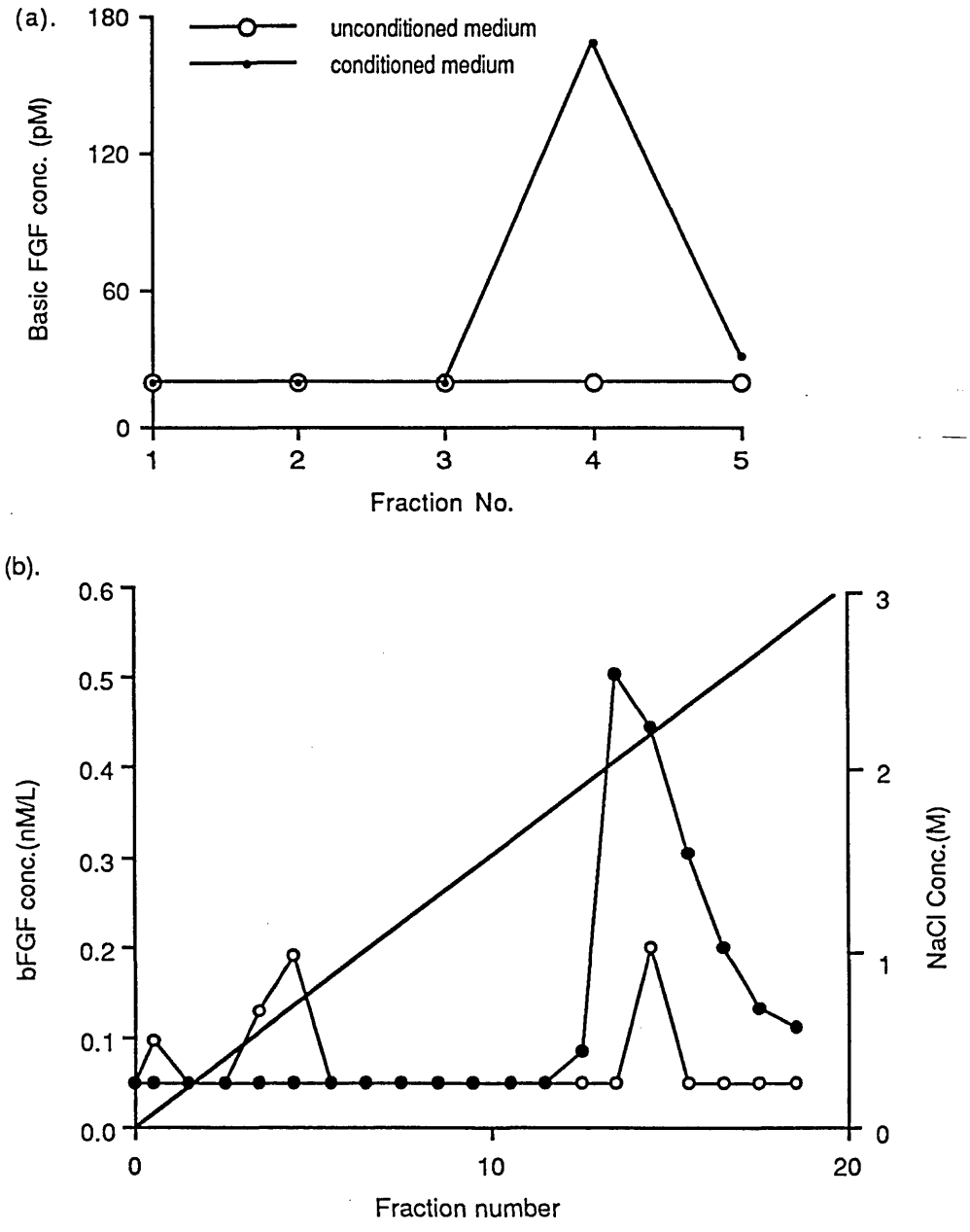


Fig. 6.1 (a) Elution of bFGF immunoreactivity in BREC-conditioned medium from a heparin-Sepharose column with 2M NaCl. (b) Heparin-Sepharose profiles of rbFGF and BREC conditioned medium

In the preliminary experiment (a), apparent bFGF immunoreactivity was not present in the fractions collected

from the column on which unconditioned medium had been adsorbed. This showed that dialysis removed NaCl from the fractions and prevented it from interfering in the RIA. The experiment also showed that bFGF immunoreactivity, present in BREC conditioned medium, could be eluted from the column with 2M NaCl.

This immunoreactivity was firmly identified as bFGF by comparing the heparin-Sepharose profiles of rbFGF and BREC conditioned medium. Recombinant bFGF was eluted at 1.9M-NaCl, reaching a peak at 2.2M. Recovery as a percentage of rbFGF applied to the column was 34.3%. BREC conditioned medium was eluted as three peaks at 0.15M-, 0.8M- and 2.3M-NaCl, the final peak corresponding to the elution position of rbFGF.

6.2.3 Cell extracts

Bovine REC were extracted by a method similar to that of Vlodavsky et al., (1987). Confluent monolayers of BREC were washed once with HBSS and trypsinized. The cells were centrifuged at 230g for 6mins and the cell pellet resuspended in Tris-HCL pH 7.4 containing 0.15M NaCl (10^6 cells/ml). The cells were disrupted by 3 cycles of freezing and thawing followed by sonication. Cell debris was removed by centrifugation at 1400g for 30 mins prior to assay. The bFGF concentration in these samples was 0.43 ± 0.25 pmol/10^6cells (mean ± s.d.; n=13; range 0.14-1.0 pmol/10^6cells).

6.2.4 ECM

Confluent cultures of BREC were washed with HBSS and 0.5% v/v Triton-X-100 in 50mM NH_4OH was added, 1ml/25cm^2flask. The alkali and detergent solubilized the cells and although some of the matrix beneath the cells was disturbed, part remained intact. The solubilized cells and debris were removed and the remaining matrix washed with HBSS. The matrix was then scraped from the plastic with a cell scraper into 10mM Tris/HCl pH 7.4 containing 2M NaCl (200μl/flask). The matrix extract was stored at -20°C. Before assay the extract was centrifuged at 1400g to remove debris and dialysed overnight

against PBSA at 4°C. The bFGF level was below the limits of detection of the RIA (20pM) in all but one sample when the level was 155 pM (31 fmol/flask).

Cell lysis releases bFGF from within the cell and this may become associated with the ECM during the solubilization procedure. To prepare ECM without cell lysis the BREC monolayer was washed with HBSS and 2M urea added. This is reported to cause cell detachment without lysis whilst leaving the ECM behind intact (Gospodarowicz et al., 1983). In our experiments however when the cells detached, the underlying matrix was pulled from the base of flask. This process was also observed during trypsinization.

6.2.5 Release of bFGF from BREC with increasing passage number

Bovine REC were maintained in culture for 14 passages in 25 cm^2 flasks and cells were split 1:2 at each passage. At each passage four flasks were washed with HBSS and 2mls of serum-free DMEM containing 0.01% w/v CHAPS was added to each flask. After 48 hrs the medium was removed, centrifuged at 1400g to remove debris and stored frozen at -20°C. The samples were assayed for bFGF using the RIA and the results are shown in Table 6.1

Passage Number	bFGF conc. pmol/10^6cells	Passage Number	bFGF conc. pmol/10^6 cells
1	0.48 ± 0.12	8	0.52 ± 0.02
2	0.74 ± 0.1	9	0.34 ± 0.02
3	0.68 ± 0.18	10	0.57 ± 0.1
4	0.56 ± 0.02	11	0.53 ± 0.05
5	0.44 ± 0.04	12	0.41 ± 0.05
6	0.52 ± 0.02	13	0.51 ± 0.05
7	0.5 ± 0.04	14	0.63 ± 0.17

Table 6.1 Release of bFGF from BREC with increasing passage number. Results are expressed as the mean ± s.d. (n=4).

6.3 Basic FGF in BREC conditioned medium measured by the monoclonal antibody based ELISA

Samples of BREC conditioned medium were assayed in 5 seperate ELISA's. The bFGF concentration was 0.024 ± 0.012 pmol/10^6cells (mean ± s.d.; range 0.006-0.034; n=7). This is a 27-fold lower concentration than that measured by RIA which was 0.65 ± 0.27 pmol/10^6cells.

6.4 Immunoblotting

Immunoblotting was carried out to answer the following questions,

1. Is the peak of immunoreactivity eluted at low salt concentrations in the BREC conditioned medium heparin-Sepharose profile due to the presence of large amounts of aFGF cross-reacting in the RIA? If true, this would also provide an explanation for the discrepancy between the high concentrations of bFGF found in BREC conditioned medium measured by RIA and the much lower concentrations seen when assayed with the monoclonal antibody ELISA.
2. Is bFGF present in extracts of ECM? More evidence was required because of the ambiguity produced by the RIA results.

The results (Fig. 6.2) show that rbFGF is detectable with anti-bFGF monoclonal antibody to 0.2pmol/μl and aFGF is similarly detectable with the anti-aFGF antibody to 0.2pmol/μl. It is also clear that aFGF cross reacts with anti-bFGF monoclonal antibody as was seen with the anti-bFGF1-24 antibody in the RIA (see Fig. 3.10).

Basic FGF is detectable in both BREC and BRP conditioned medium but only very faint colour development is seen with anti-aFGF antibodies. There is also evidence of the slight cross-reactivity of aFGF antibodies with rbFGF. Therefore, if aFGF is present in BREC conditioned medium it is only present in small amounts and could not account for the peak of immunoreactivity seen in the BREC conditioned medium heparin-Sepharose profile or the discrepancy between the bFGF

measurements in the RIA and the ELISA. Basic FGF is detectable in the ECM extract.

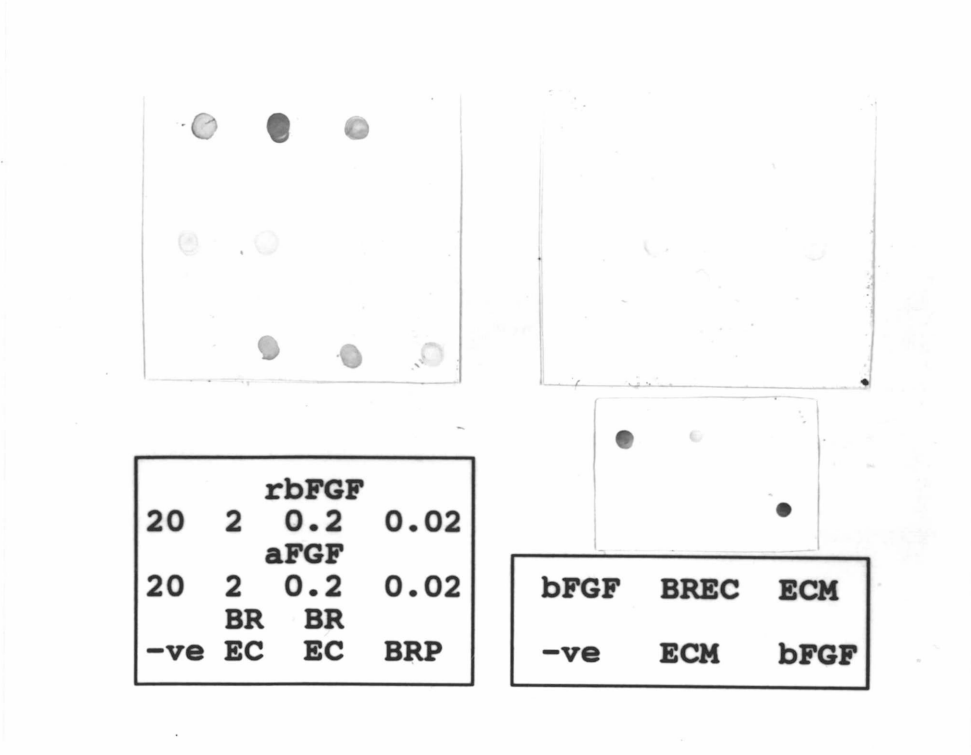


Fig. 6.2. Immunoblotting of recombinant bFGF, purified aFGF, BREC and BRP conditioned medium with anti-bFGF monoclonal antibody (top left) and anti-aFGF antibody (top right). The key is at bottom left with growth factor concentrations in pmol/μl. A blot of ECM extract probed with anti-bFGF antibody is also shown (lower right) with its key below.

6.5 Characterization of release of bFGF from BREC: Relationship to cell damage

6.5.1 Introduction

The aims of the study were (1) to examine the pattern of bFGF release from BREC with time, (2) to relate this release to parameters of cell damage and lysis, and (3) to measure its bioactivity using methyl-^{3}H thymidine incorporation.

6.5.2 Methods

Growth factor release from BREC monolayers with time

Sub-confluent bovine retinal endothelial cells were treated with trypsin (0.25% w/v) and a single cell suspension produced by passing cell clumps through a 21 gauge needle. An equal volume of this suspension was then added to each of twenty four 25 cm^2 tissue culture flasks previously treated with fibronectin and the cells grown in DMEM containing 10% PDS. At confluence the medium was removed and the monolayer washed with HBSS. 2 mls of serum-free DMEM containing 0.01% w/v CHAPS was added to each flask and the flasks sealed and incubated at 37°C. 21 flasks were used for measuring cumulative growth factor release over a period of 6 days and 3 for measuring daily release for 6 days from the same monolayer and subsequent results and discussion will refer to either serum-free cumulative or daily release. Photographs were taken of the cells every day (Fig. 6.4). A further series of experiments were carried out on twenty one, 25 cm^2 flasks containing cells prepared identically to those above. At confluence the medium was removed , the monolayer washed with HBSS and 2mls of DMEM containing 5% v/v human serum (North London Blood Transfusion Centre) was added to each flask and the flasks sealed and incubated at 37°C. These flasks are referred to as serum-containing cumulative release flasks. At time = 0 and every day for 6 days, 3 of the serum-free cumulative release flasks, 3 of the serum-containing cumulative release flasks and the 3 flasks used for measuring daily release had their medium removed and centrifuged at 670g for 10 minutes. The medium from each flask was divided into four 0.5ml aliquots and these were stored frozen at -20°C prior to growth factor assays. Aliquots for growth factor bioassay were prepared under sterile conditions using medium removed from the serum-free cumulative release flasks. The remaining pellet of debris and cells was resuspended in 0.75mls of HBSS and counted with a Coulter Counter. 2mls of fresh serum-free DMEM containing 0.01% CHAPS was added to the three flasks used for measuring daily release. Medium and

debris was removed from these flasks each day and fresh serum-free DMEM and 0.01% CHAPS added. The cell monolayer in the 3 serum-free and serum-containing cumulative release flasks was washed with HBSS and treated with 1ml of 0.25% w/v trypsin until all cells had detached. The cells were resuspended in 1ml of HBSS and counted.

Growth factor release from lipopolysaccharide-treated and sub-confluent BREC monolayers

Cell growth conditions were chosen in order to achieve minimum (sub-confluent growing cells) and maximum (addition of lipopolysaccharide) cell damage. BREC were plated into 25cm^2 flasks at either high density (600,000 cells per flask) or low density (200,000 cells per flask). The cells in the flasks seeded at high density were grown to confluence whereas those seeded at low density were allowed to attach overnight at 37°C (sub-confluent flasks). Medium was then removed and the cells washed with HBSS. 2mls of serum-free DMEM containing 0.01% CHAPS or 2mls of DMEM containing 5% human serum was added to the flasks containing the confluent cells and lipopolysaccharide from E.coli 55:B5, (10 μgml^{-1}) (Sigma) was added to half the flasks in each group. 1ml of serum-free DMEM containing 0.01% CHAPS or 1ml of DMEM containing 5% human serum was added to the flasks containing the sub-confluent cells to achieve similar medium to cell ratios as used for the confluent cells. All flasks were incubated at 37°C for 24 hours. The medium was then removed and aliquoted and the cells trypsinized and counted as in the cumulative growth factor release experiments above except that the medium from the sub-confluent flasks was split into 0.25ml aliquots. Aliquots were stored at - 20°C prior to growth factor assays.

Assay for lactate dehydrogenase (LDH)

LDH was measured kinetically on an RA-XT Analyser (Technicon Ins. Corp., Basingstoke, U.K.) using a spectophotometric method optimized to Scandanavian conditions

(pyruvate to lactate) at 340 nm (The Committee on Enzymes of the Scandanavian Society for Clinical Chemistry and Clinical Physiology, 1974).

6.5.3 Results

Effect of serum-free and serum-containing DMEM on BREC monolayers

A confluent monolayer of BREC contained 6.8 ± 0.4 (x 10^5) cells per 25 cm^2 flask (mean ± SEM; n = 3). After 24 hours in serum-free medium this number had dropped to 5.2 ± 0.6 (x 10^5) cells per flask (Fig. 6.3). This decrease continued to 4.1 ± 0.5 (x 10^5) cells per flask by day 6.

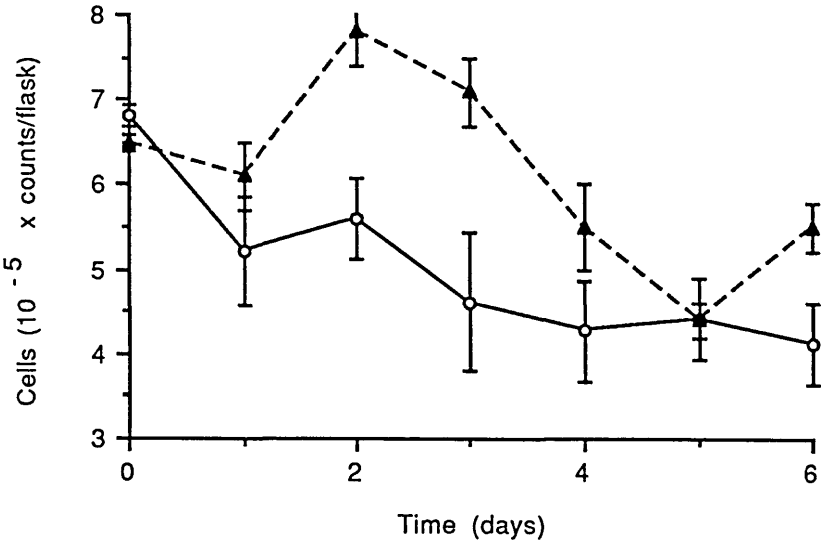


Fig. 6.3 Change in BREC cell number with time in serum-free (○) or serum-containing (▲) culture medium (mean ± SEM; n=3)

The photographic record of the BREC monolayer at T=0 and at day 2, 4 and 6 after the addition of serum-free DMEM and 0.01% CHAPS is shown in figure 6.4(a-d). At T=0 the confluent monolayer shows closely packed cells with some cells rounded up on the surface of the monolayer, these appear as bright beads in the phase contrast micrographs. By day 2 there is an

increase in the number of rounded cells in the monolayer and some of these are seen floating in the medium. Also several smaller beads and many small dark particles can be seen, these are the components of cell debris. On day 4 the monolayer appears severely disrupted, with many dead cells and cell debris particles, however at this stage it is predominantly intact. Finally on day 6 there is the complete break up of the monolayer with large gaps between the endothelial cells and an increased amount of cellular debris.

Confluent cell monolayers in medium containing serum showed a different pattern of changes in monolayer cell number compared to that seen in serum-free medium. There was an initial drop from 6.5 ± 0.1 (x 10^5) to 6.1 ± 0.4 (x 10^5) cells per flask but the number then increased to 7.8 ± 0.4 (x 10^5) cells per flask after 48 hours before falling to reach the levels seen in serum-free medium by day 5. The early increase could be explained by unsustained cell proliferation in response to released growth factors. Monolayer disruption as viewed through the microscope, was similar to that seen in serum-free medium.

Release of cells from the monolayer and increase in cell debris in serum-free and serum-containing culture medium

There was an increase in the number of cells present in serum-free culture medium as measured with the Coulter Counter (Fig. 6.5) which coincided with a decrease in cell number in the BREC monolayer . There was a greater rate of increase in particles counted in the medium, 5.2 ± 0.2 x (10^5) counts per flask per day over the first 4 days compared to the decrease of cells in the monolayer of 0.57 ± 0.15 x (10^5) cells per flask per day over the same period. There was no further decrease in monolayer cell number over the last 2 days whereas the cell and debris count in the medium continues to increase by 2.3 ± 0.9 (x 10^5) counts per flask per day. These results could be partly explained by cell proliferation in the monolayer but there is also break up of cells into smaller fragments as seen in the photographic record.

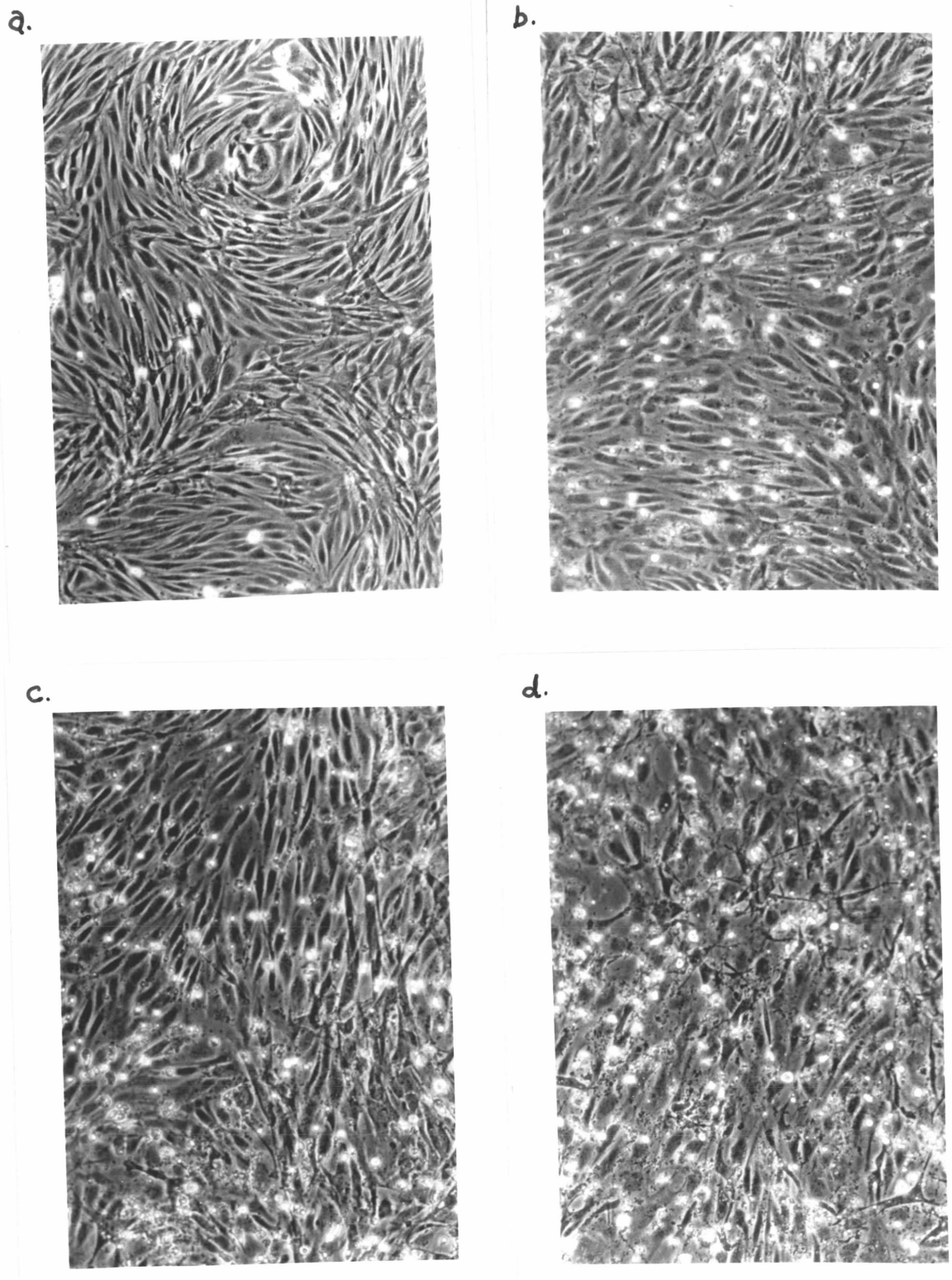


Fig 6.4 Changes in the appearance of confluent monolayers of BREC in serum-free DMEM with time. Flasks of confluent BREC used for the serum-free cumulative growth factor release experiments were photographed daily over the period of the experiment. Results are shown for days 0 (a), 2 (b), 4 (c) and 6 (d) after addition of serum-free medium. Magnification x 50

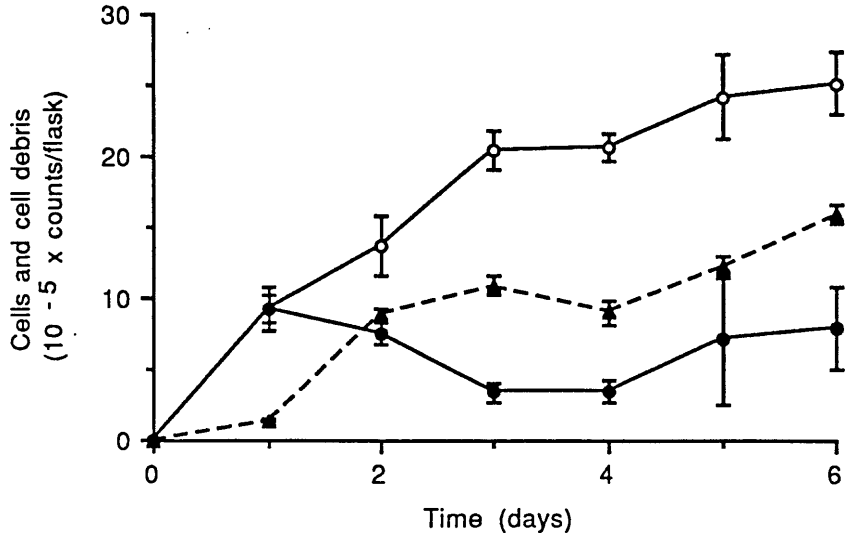


Fig. 6.5 Cells and cell debris released into the culture medium in serum-free cumulative release flasks (O), daily release flasks (•) or serum-containing cumulative release flasks (▲) (mean ± SEM; n=3)

Cells and cell debris particles measured in serum-containing culture medium increased at a lower rate (2.6 ± 0.6 (x 10^5) counts per flask) than in serum-free medium and this could be explained by less cell damage. Monolayer cell number however, eventually decreased to the levels seen in serum-free medium indicating that similar monolayer damage did occur in serum-containing medium and a better explanation is that there was less break up of released cells into smaller fragments. The number of cells remaining in the BREC monolayer in those flasks where serum-free medium had been removed and replaced daily was 4.0 ± 0.7 (x 10^5) cells per flask.

Basic FGF release into serum-free and serum-containing culture medium

Basic FGF release from BREC monolayers is shown in Fig. 6.6. The results are expressed as a percentage of the maximum release of bFGF in each experiment, since the release of bFGF from BREC monolayers varied depending on the batch of cells used.

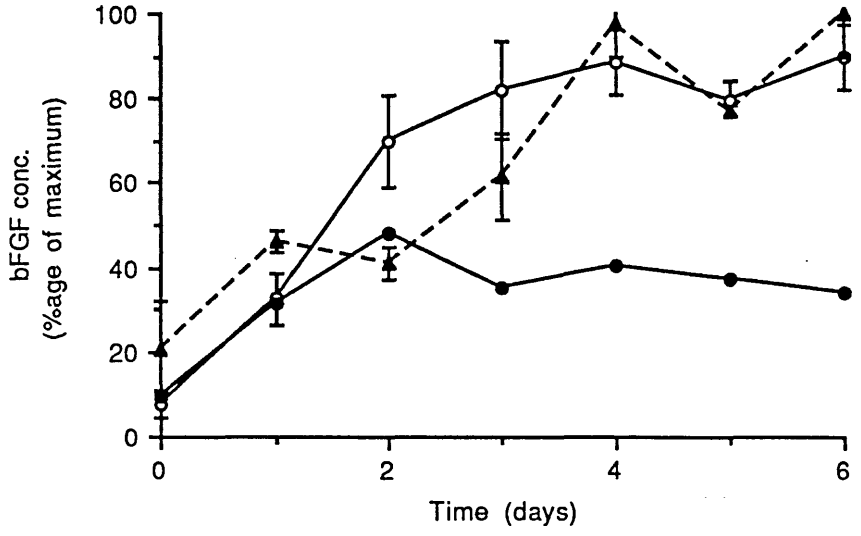


Fig. 6.6 Release of bFGF into the culture medium. Results are shown as a percentage of the maximum release for each experiment in serum-free cumulative release flasks (O), daily release flasks (•) or serum-containing cumulative release flasks (▲) (mean ± SEM; n=3 except for daily release flasks where n=2)

The rate of release of bFGF over the first 4 days in each of the 3 experiments was 31.8, 71.0 and 105.2 $pMday^{-1}$ in the serum-free cumulative release flasks reaching a maximum of 149, 476 and 467 pM respectively. Basic FGF was released into the serum-containing culture medium at rates of 49.0, 61.3 and 54.3 $pMday^{-1}$ over the first 4 days and reached a maximum of 224, 280 and 315 pM respectively. Basic FGF was released at rates of 49 $pMday^{-1}$ and 177 $pMday^{-1}$ in the daily release flasks.

IGF-1 release into serum-free and serum-containing culture medium

The results for IGF-1 release are shown in figure 6.7.

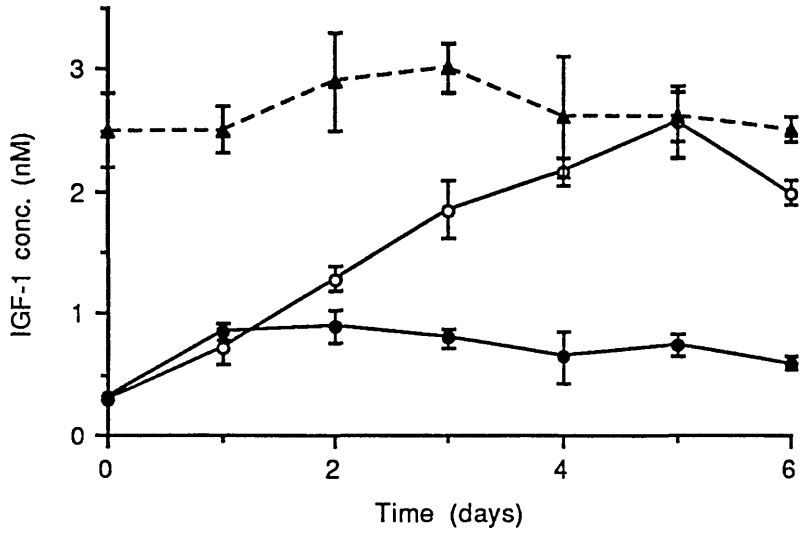


Fig 6.7 Release of IGF-1 into the culture medium in serum-free cumulative release flasks (O), daily release flasks (•) or serum-containing cumulative release flasks (▲) (mean ± SEM; n=3)

IGF-1 was released into serum-free DMEM by BREC monolayers at a rate of 0.34 ± 0.02 nMday^{-1} reaching a maximum of 2.56 nM. The release of IGF-1 in the daily release flasks was 0.75 ± 0.05 nMday^{-1}. A baseline level of IGF-1 of 2.50 ± 0.28 nM, due to the presence of IGF-1 in serum, was seen in serum-containing culture medium and there was no significant increase in IGF-1 concentration over the time course of the experiment.

LDH release into serum-free and serum-containing culture medium

The results for LDH release are shown in figure 6.8. All results are the mean ± SEM of data from 3 separate experiments. LDH release into serum-free DMEM by BREC followed a rate of 105 IUl^{-1}day^{-1} to reach a maximum of 628 IUl^{-1}. A constant release of 90 ± 7 IUl^{-1}day^{-1} was seen in the same monolayer when fresh medium was added daily. LDH was released into serum-containing medium at a rate of 110 IUl^{-1}day^{-1} reaching a maximum of 689 IUl^{-1}.

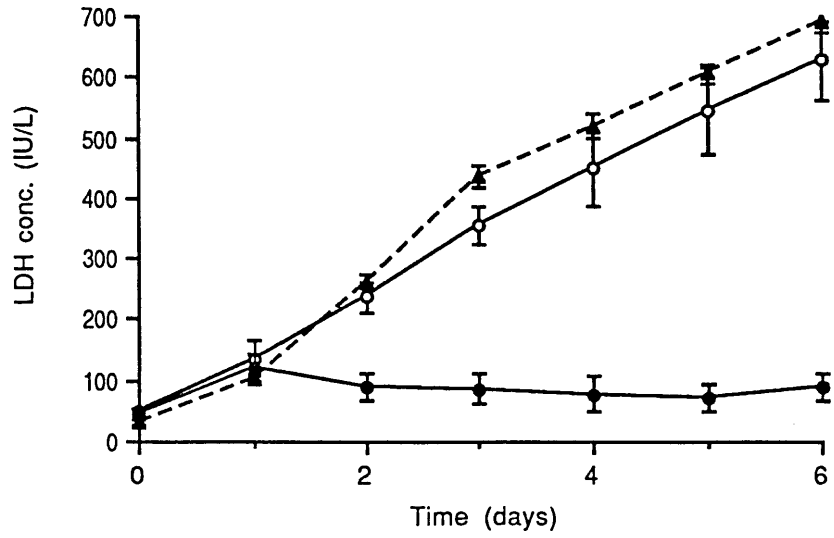


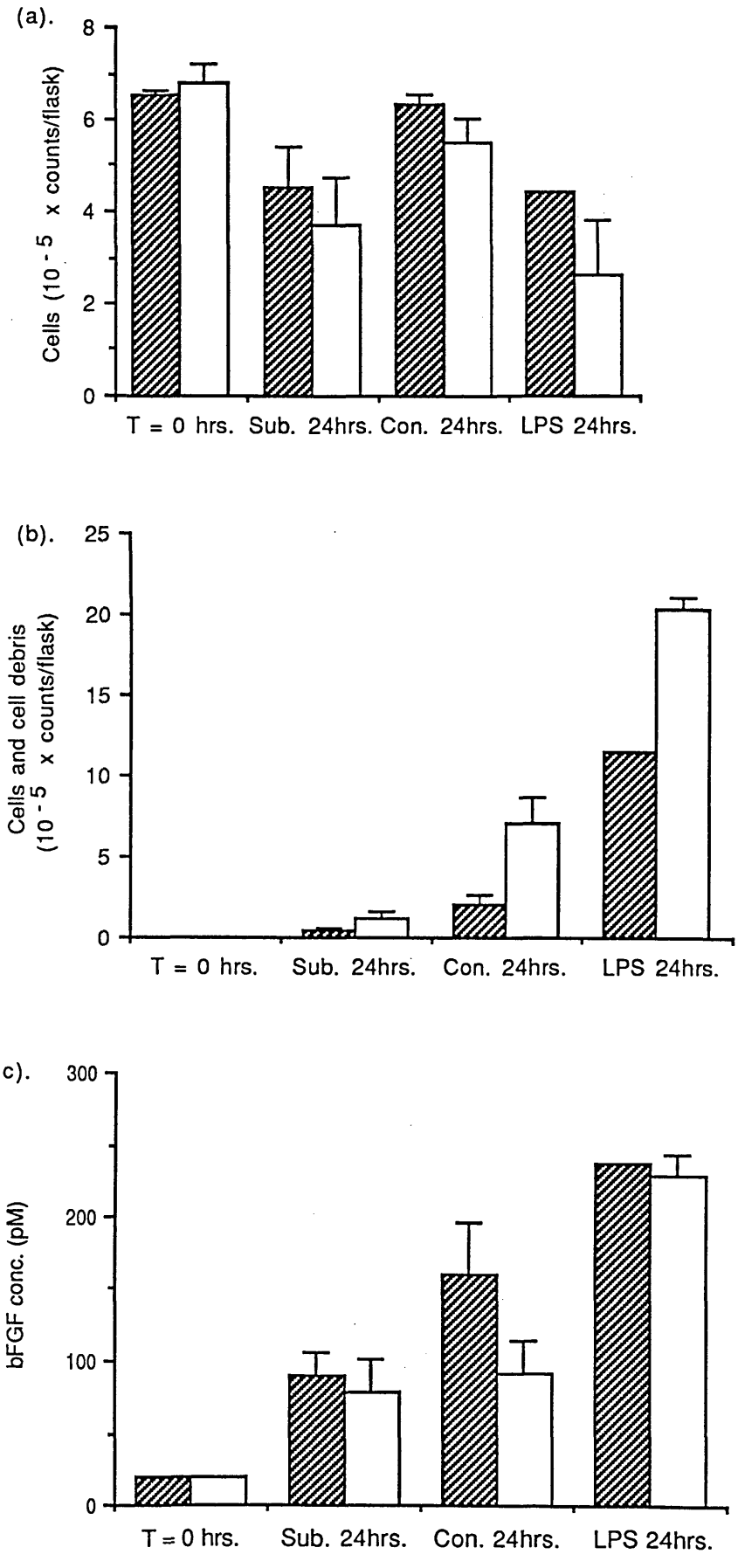
Fig. 6.8 Release of LDH into the culture medium in serum-free cumulative release flasks (O), daily release flasks (•) and serum containing cumulative release flasks (▲) (mean ± SEM; n=3)

This was similar to the pattern of release seen in serum-free medium indicating a comparable amount of cell lysis in cell monolayers maintained with or without the addition of serum.

Growth factor release from lipopolysaccharide-treated and sub-confluent BREC monolayers

Basic FGF release, after 24 hours, from sub-confluent BREC and confluent cells with or without lipopolysaccharide (10 $\mu g ml^{-1}$) into both serum-free and serum-containing medium is shown in Figure 6.9. Also shown are the corresponding changes in IGF-1 concentration, LDH concentration , cell and cell debris particles in the medium and monolayer cell number. All columns represent the mean ± SEM of data from at least 3 experiments except where indicated.

Fig. 6.9 (a-c)


(a).
Cells (10 - 5 x counts/flask)
0
2
4
6
8
T = 0 hrs.
Sub. 24hrs.
Con. 24hrs.
LPS 24hrs.
(b).
Cells and cell debris (10 - 5 x counts/flask)
0
5
10
15
20
25
T = 0 hrs.
Sub. 24hrs.
Con. 24hrs.
LPS 24hrs.
(c).
bFGF conc. (pM)
0
100
200
300
T = 0 hrs.
Sub. 24hrs.
Con. 24hrs.
LPS 24hrs.

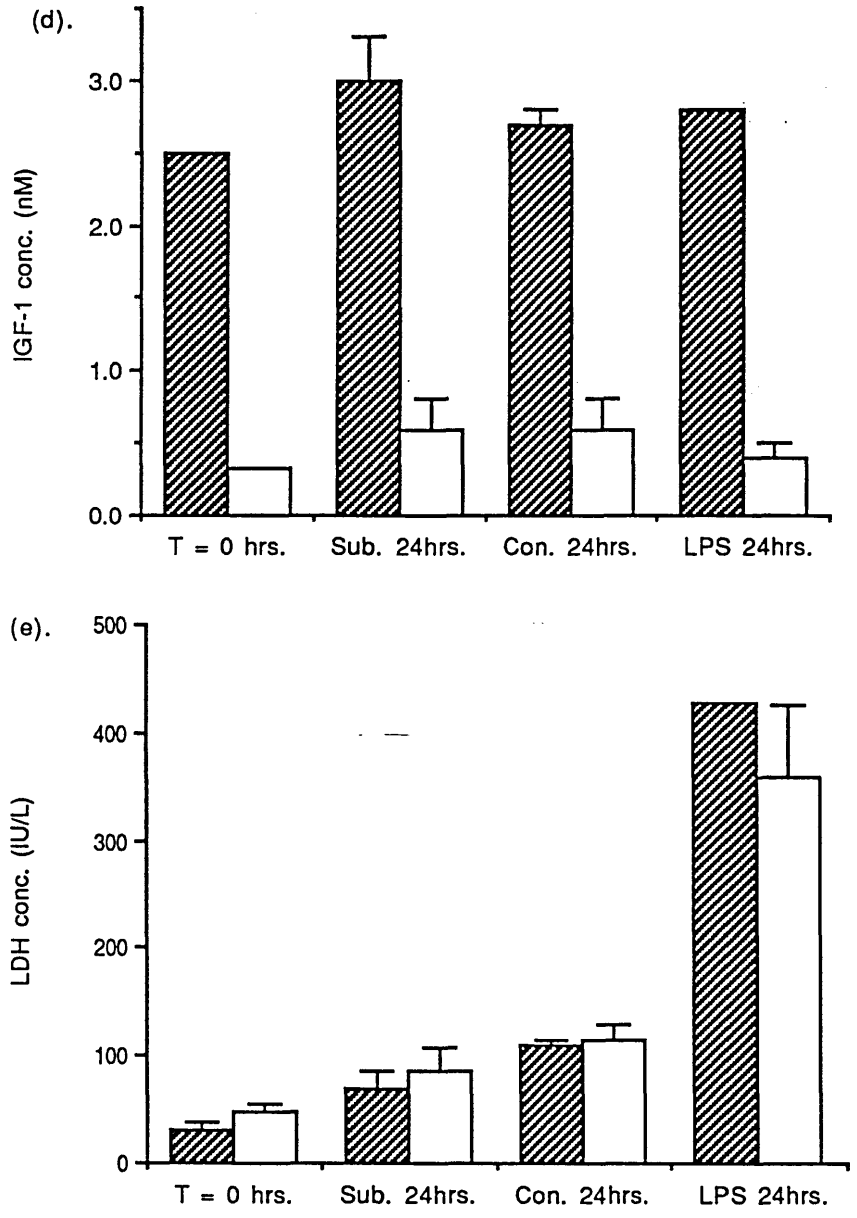


Fig. 6.9 Growth-factor release and changes in parameters of cell damage in sub-confluent BREC monolayers and confluent monolayers treated with lipopolysaccharide. Results shown are the means for (a) monolayer cell number, (b) cell and cell debris particle numbers, (c) bFGF concentration, (d) IGF-1 concentration and (e) LDH concentration in serum-free medium (□) or serum containing medium (■) at zero time (t=0) and at 24hrs in flasks containing sub-confluent cells (Sub. 24 h), confluent cells (Con. 24 h) or confluent cells treated with lipopolysaccharide (LPS 24 h). (mean ± SEM for at least 3 separate experiments , except for results from serum-containing confluent cells at 24hrs and treated with lipopolysaccharide where two and one experiments were carried out respectively

Basic FGF concentrations were 91 ± 24 pM in serum-free medium and 161 ± 35 pM in medium containing serum. These were increased to 230 ± 14 and 238 pM respectively when the cells were treated with lipopolysaccharide (10 μgml^{-1}). The decrease in monolayer cell number and increase in cell and cell debris particles measured in the medium were also greatest when the cells were treated with lipopolysaccharide. The number of cell and cell debris particles measured in serum-containing medium after 24 hours was less than that seen in serum-free medium. This was also observed in the cumulative release experiments and was probably due to less cell break up in the presence of serum. LDH release, however, was similar into both serum-free (116 ± 13 IUl^{-1}) and serum-containing (109 ± 5 IUl^{-1}) medium and was substantially increased after the addition of lipopolysaccharide to 360 ± 56 and 428 IUl^{-1} respectively. These results suggest that although the amount of cell debris was lower in serum-containing medium, cell lysis, as measured by LDH release, was just as great. IGF-1 concentrations did not show any change in serum-containing medium from the baseline level of 2.50 ± 0.28 nM and there was no increase when the cells were treated with lipopolysaccharide.

Basic FGF was released into serum-free and serum-containing medium by sub-confluent BREC, reaching levels of 85 ± 23 and 70 ± 16 pM respectively. Cell number at 24 hours was similar at 3.7 ± 1.0 and 4.5 ± 0.9 (x 10^5) cells per flask and although there were few cell and cell debris particles in the medium (1.2 ± 0.7 and 0.5 ± 0.1 (x 10^5) counts per flask), LDH release, (85 ± 23 and 70 ± 16 IUl^{-1}) was similar to that seen from confluent cells. This indicates that cell lysis had occurred and this could account for the release of bFGF. There was no significant release of IGF-1 from subconfluent cell monolayers.

6.5.4 Bioactivity of BREC conditioned medium

Sub-confluent BREC were treated with 0.25% trypsin and a single cell suspension prepared as previously described. The cells were counted with a Coulter Counter and seeded into

each well of 24-multiwell tissue culture plates, pre-coated with fibronectin, at 2500 cells per well. Methyl-^{3}H thymidine incorporation was carried out using the optimized method (see Section 2.6.2). Wells were treated individually by the addition of either 100 μl of serum-free BREC conditioned medium harvested from BREC monolayers at days 0 to 6 as described earlier or bFGF (5 pM).

Addition to cell-culture well	Methyl-^{3}H thymidine uptake(dpm)
100μl BREC conditioned medium harvested at 0 days	7550 ± 479
1 day	10467 ± 924
2 days	11307 ± 783
3 days	10210 ± 543
4 days	11171 ± 985
5 days	12075 ± 760
6 days	10073 ± 417
rbFGF (5pM)	10678 ± 343

Table 6.2 Bioactivity of BREC conditioned medium. Results are means ± SEM (n=6)

Medium from serum-free cumulative release flasks sampled on days 1 to 6 produced significantly increased methyl-^{3}H thymidine uptake into BREC when compared to medium sampled at T=0, see Table 6.2. Basic FGF (5 pM) also showed significant stimulation of methyl-^{3}H thymidine incorporation. Values were compared by one-way ANOVA ($p<0.01$)and then each value was compared with that at zero time by an unpaired t test ($p<0.05$ in every case).

6.6 Basic FGF mRNA in bovine retinal endothelial cells and human astrocytoma cells

6.6.1 Experiment 1 - RNA from cells grown in medium containing different oxygen tensions

Two batches of second passage BREC were grown until sub-confluent in 4 x 175 cm^2 flasks. In one flask of each pair the medium was replaced with degassed medium and the flasks flushed with 95% nitrogen:5% CO_2. This procedure reduced the oxygen tension in the medium as described in Section 7.4.1 and these flasks were called low O_2 flasks (63 mmHg; n=2) The other flask of each pair had medium replaced with standard culture medium and the flasks were flushed with 95% air:5% CO_2. These flasks are referred to as high O_2 flasks (152 mmHg; n=2). All flasks were incubated for a further 48hrs. The cells (approximately 6 x 10^6 cells/flask) were then harvested and total RNA was prepared from each flask. On a separate occasion 4 x 75 cm^2 flasks of confluent human astrocytoma cells were harvested and total RNA was prepared.

After resuspending the RNA in 100μl of DEPC-treated distilled water the quantity and purity of the RNA was estimated and the results are shown in Table 6.3

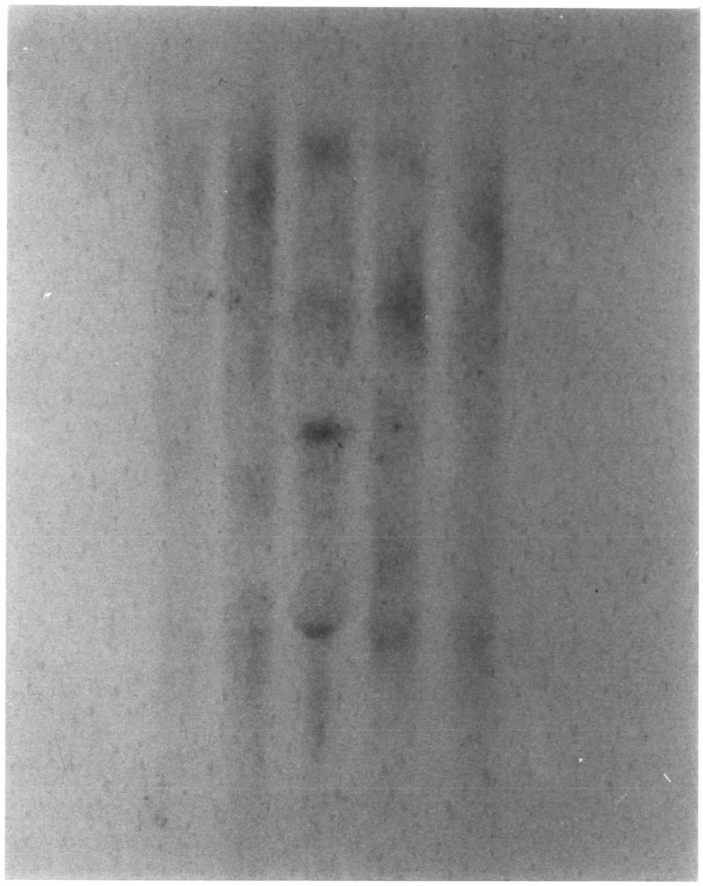
Treatment group	Purity O.D.260/280nm	Quantity (μg/100μl)	Volume used for blot(μl)
Astrocytoma	1.82	190	5
Bx.1 High O_2	2.14	4.8	65
Bx.1 Low O_2	1.36	6.1	51
Bx.2 High O_2	1.89	17.0	18.5
Bx.2 Low O_2	8	2.6	95

Table 6.3 Purity and quantification of RNA from experiment 1.

The volumes shown in Table 6.3 were evapo rated in a vacuum centrifuge until almost dry, mixed with loading dye

(see Section 2.7.6) and loaded onto the gel and. Electrophoresis was carried out for 3hrs. at 200V. The gel was blotted overnight and baked at 80°C for 2hrs.

The bFGF cDNA probe was labelled to a specific activity of 1.2 x 10^8 cpm/μg. The blot was prehybridized at 55°C and 3 x 10^5 cpm of probe per ml of hybridization buffer was added and the blot incubated for 18hrs at 55°C. The blot was washed twice with 6 x SSC, twice with 2 x SSC and once with 1 x SSC at 55°C. The film was exposed to the blot for 4 days at -70°C and the resulting autoradiograph is shown in Fig. 6.10.



1 2 3 4 5

Fig. 6.10 Autoradiograph of a Northern blot of BREC and astrocytoma RNA hybridized to a bFGF cDNA probe. Lane 1 = Bx.2 Low O_2, Lane 2 = Bx.2 High O_2, Lane 3 = Astrocytoma, Lane 4 = Bx.1 High O_2 and Lane 5 = Bx.1 Low O_2

The astrocytoma lane shows 2 distinct bFGF mRNA transcripts with another less well defined higher molecular weight transcript. These bands are probably equivalent to the 7.0, 3.7 and 1.8kB transcripts described by Murphy et al., (1988). The lanes of BREC RNA show indistinct areas of hybridization that approximately correspond to the positions of the astrocytoma bands. It is clear that significant degradation of the RNA occurred in these samples probably due to a failure to completely eliminate RNAases. No information could be obtained on the effect of low oxygen tension on the levels of these transcripts.

6.6.2 Experiment 2 - The effect of phorbol ester, cycloheximide and serum on bFGF mRNA transcripts

Thirty six 175cm^2 flasks of 2nd passage BREC were incubated until the cells were approximately 50% confluent. They were divided into six groups of 6 flasks and the groups were treated as follows:-

1. Cycloheximide (10μM) added for 2hrs.
2. No additions

Fresh DMEM + 10% v/v PDS was added to the following flasks 24 hrs before the treatments as follows :-

3. TPA (1μM) added for 4hrs with cycloheximide (10μM) added for the last 2hrs.
4. TPA (1μM) was added for 4hrs.
5. Cycloheximide (10μM) was added for 2hrs.
6. No additions

The cells were harvested and RNA prepared. The RNA was quantified and the purity assessed with the results shown in Table 6.4

Molecular weight markers were prepared by 5′ end-labelling of DNA fragments from a HindIII digest of bacteriophage lambda DNA to a specific activity of 10^6cpm/μg.

RNA within each treatment group was pooled and 20μg of

RNA from each group, in the volumes shown in Table 6.4, were loaded onto a 1.2% agarose gel alongside 20μg of RNA from astrocytoma cells prepared as described in experiment 1 and 7μl (31,250 cpm) of the molecular weight markers. Electrophoresis was carried out at 200V for 4.5hrs. The gel was blotted overnight, baked at 80°C for 2hrs., and prehybridized.

Treatment group	Purity O.D.260/280nm	Quantity (μg/400μl)	Volume used(μl)
1	2.01	68	118
2	1.73	43μg/200μl	93
3	1.83	131	61
4	1.70	149	54
5	1.64	117	68
6	1.77	102	79
Astrocytoma	1.82	190μg/100μl	10.5

Table 6.4 Purity and quantification of RNA from experiment 2

Basic FGF cDNA probe, labelled to a specific activity of 2.8×10^8 cpm/μg was added to the blot (7×10^5 cpm/ml of hybridization buffer) and incubated for 18hrs at 60°C. Stringency washes were twice at 6 x SSC and twice at 1 x SSC, both at 55°C. The film was exposed to the blot for 18hrs at -70°C and the autoradiograph is shown in Fig. 6.11.

The blot was stripped and rehybridized to a rat actin oligonucletide probe (6×10^5 cpm/ml) labelled to a specific activity of 2.9×10^8 cpm/nmol.

The astrocytoma lane shows hybridization to 4 areas coresponding to 7.0, 3.7, 1.8 and 1kb. The bands are not well defined indicating some degradation by RNAases. Four of the BREC mRNA lanes show hybridization to mRNA transcripts. The fresh serum with no further additions lane shows the

strongest signal with transcripts at 7.0, 3.7 and 1.0kb. The serum starved with no other additions and the fresh serum and TPA lane both show transcripts at 3.7 and 1.0kb with faint evidence of a band corresponding to 7.0kb. The serum starved, cycloheximide-treated lane shows one band at 7.0kb however the other lanes of RNA from cells treated with cycloheximide show no hybridization. Hybridization to the rat actin oligonucleotide was low but was detectable on the autoradiograph. Whether this was due to the specific activity of the probe being too low, the hybridization conditions being too stringent or poor hybridization of this rat actin probe to bovine and human actin mRNA is not clear. The results do however show the least hybridization to the lanes of RNA from BREC treated with fresh serum and cycloheximide possibly explaining the absent hybridization signal to the bFGF cDNA probe in these lanes.

The hybridization bands in this study were not very distinct indicating both digestion of the RNA by RNAases and low specific activity of the probe. It was not possible to obtain any information about the effect of the treatments on levels of the bFGF mRNA transcripts. However, these results are the first demonstration that it is possible to detect mRNA transcripts for bFGF using total RNA without Oligo-dT cellulose purification of mRNA.

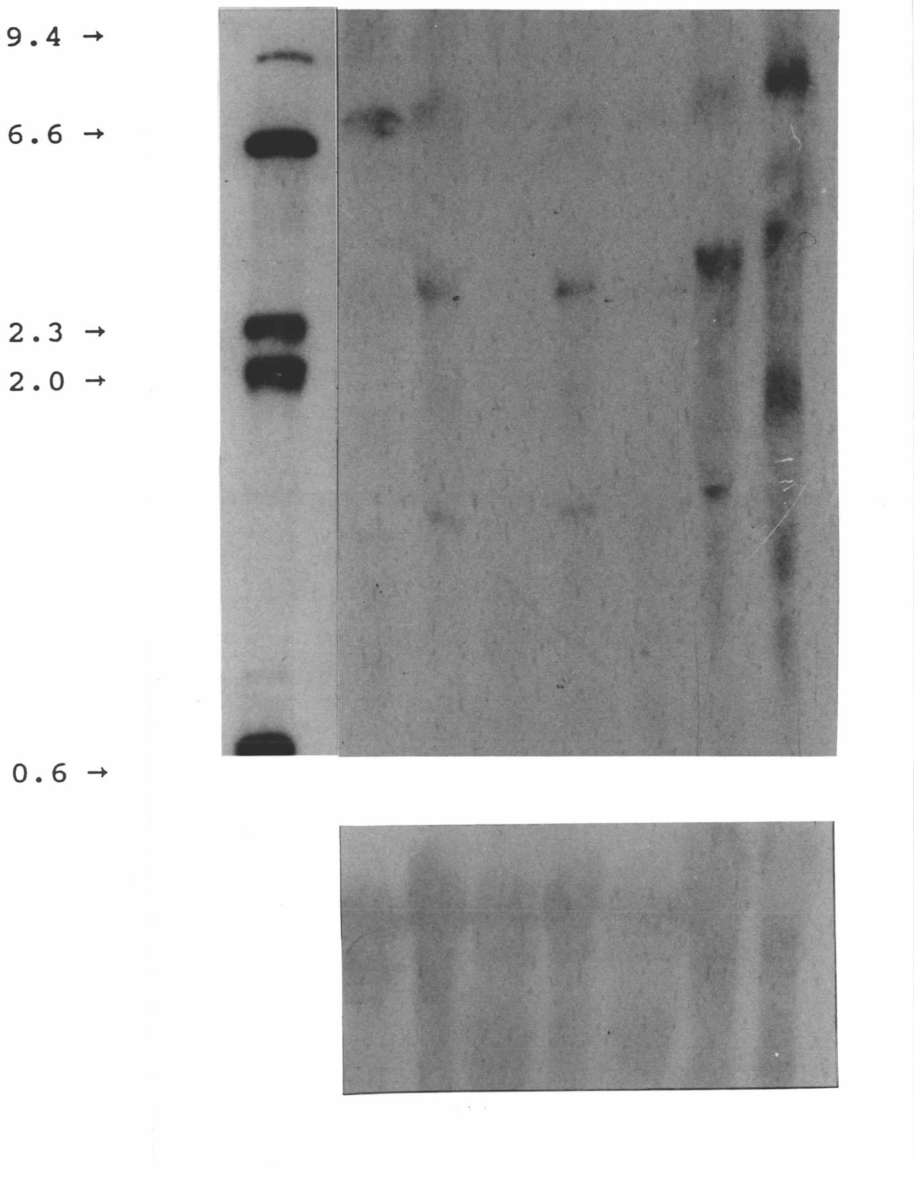


Fig. 6.11 Autoradiographs of RNA from BREC and astrocytoma cells. The upper panel shows 6 lanes of BREC RNA (for cell treatments see the key at the beginning of this section) and one lane of astrocytoma RNA (A), hybridized to bFGF cDNA. Labelled molecular weight markers are shown alongside. The lower panel shows an autoradiograph of the same blot hybridized to a rat actin oligonucleotide probe. MW = molecular weight markers with sizes shown in Kb.

6.7 Discussion

We have been able to demonstrate the presence of bFGF in culture medium conditioned by BREC using RIA. This confirms the results of Bensaid et al., (1989a) who used bioassays and western blotting to measure and characterize bFGF. The levels released from BREC (0.65 ± 0.27 pmol/10^6 cells) are equivalent to a concentration of 325pM in the medium. These levels are very high when compared to the ED_{50} value of 2.8pM for stimulation of BREC proliferation by rbFGF. These high concentrations could however only be measured when a minimum volume of medium was conditioned by a confluent monolayer of BREC. During the 48 hours over which the medium was conditioned, many cells rounded up and were released from the monolayer into the medium. It was initially thought that this damage to the monolayer was due to the use of serum-free medium however subsequently similar changes were seen to confluent monolayers in medium containing serum. A comparable phenomenon has been reported in post-confluent cultures of bovine aortic endothelial cells in the absence of exogenous bFGF (Gospodarowicz et al., 1987a; Speir et al., 1991). The measurement of bFGF in medium conditioned by confluent cultures led to the idea that cell damage could be responsible for the release of the large quantities of this growth factor.

The results from the measurement of bFGF in medium conditioned by cells of increasing passage number indicate that even late passage BREC continue to produce a similar amount of this growth factor. It is possible that bFGF is responsible for maintainance of the differentiated phenotype seen in these cells as has been described for other endothelial cells (Gospodarowicz et al., 1987a).

The extracted cells showed that bFGF was present intracellularly although at a concentration which suggested that all the available bFGF was released from the cells during the preparation of conditioned medium. These levels could also be explained by some of the intracellular bFGF remaining associated with cellular membranes during the preparation of the cell extract.

In order to study the bFGF content of ECM it was necessary to prepare matrix without adherent endothelial cells because of the high concentrations of bFGF which they contain. This preparation was very difficult and we found that the methods used by others, (Vlodavsky et al., 1987a), resulted in the adherence of ECM to the cells. There have been no reports of the preparation of ECM from retinal endothelial cells, other authors have used bovine corneal, aortic and adrenal endothelial cells (Gospodarowicz et al., 1986b; Baird & Ling, 1987; Vlodavsky et al., 1987a; 1987b) and although the matrix produced by BREC has been studied (Canfield et al., 1986) it was not separated from the endothelial cells during preparation. It is possible that endothelial cells from other sources produce an ECM which is more adherent to the tissue culture plastic or less adherent to the endothelial cells. Vlodavsky et al., (1987a) had similar difficulties in matrix preparation from bovine aortic endothelial cells and used ECM produced by bovine corneal cells for their studies. These investigators found that bovine corneal endothelial cell ECM remained firmly adherent to the tissue culture plastic even after extensive washing.

We found that it was possible to lyse the cells with detergent and wash away the resulting debris whilst leaving some of the matrix behind. Basic FGF was detected by RIA in one of these preparations although the concentrations were very low. The presence of bFGF in the ECM was confirmed by immunoblotting. Whether bFGF was already present in the ECM or became associated with it after cell lysis is not clear. Basic FGF concentrations in the ECM of bovine aortic endothelial cells were found to be very low unless the cells were lysed during preparation (Gajdusek & Carbon, 1989). These authors proposed that bFGF becomes associated with the matrix following release from lysed cells and that the small amounts present in the ECM prepared from non-lysed cells was due to cell death and turnover or to a small amount of cell lysis produced by routine manipulation of the culture. A mechanism has also been described for the release of bFGF and its association with ECM following transient disruptions to the cell plasma membrane during normal cell growth (McNeil et

al., 1989). However, it is also proposed that bFGF is deposited in ECM without the requirement for cell lysis. Vlodavsky et al., (1987a) demonstrated bFGF in bovine corneal endothelial cell ECM which was prepared without lysing the cells and Rogelj et al., (1989) showed that the clonal growth of bovine aortic endothelial cells, which required bFGF in the ECM, was supported by matrix prepared by lysing confluent monolayers of bovine aortic endothelial cells in the presence and absence of heparin which would compete with the matrix heparan sulphate proteoglycans for binding released bFGF. These researchers also transfected a mouse endodermal carcinoma cell line with a bFGF expression plasmid and showed that ECM produced by transfected cells but not by non-transfected cells supported bovine aortic endothelial cell clonal growth. When confluent densities of bovine aortic endothelial cells were seeded onto matrix produced by non-transfected carcinoma cells and then lysed after cell attachment and spreading, the resultant matrix would not support the clonal growth of vascular endothelial cells indicating that bFGF does not rapidly associate with the ECM after cell lysis.

The lower concentrations of bFGF in BREC conditioned medium measured using the monoclonal antibody based ELISA raises questions about the nature of the bFGF measured in the medium by RIA. The 27-fold lower concentrations could be due to a number of reasons. The ELISA has an increased specificity over the RIA and requires the binding of two antibodies to two separate epitopes on the bFGF molecule. Levels of growth factor measured in the ELISA would be lower if the majority of the bFGF in BREC conditioned medium were in a degraded form so that it could still compete for antibody binding in the RIA but not be able to bind to two antibodies in the ELISA. Measured levels of bFGF would also be low if it were bound to another molecule which masked one of the antigenic sites. There may be conformational differences between the rbFGF used for preparing the standards and the native bFGF released from the cells which allow the former to present epitopes to two antibodies whilst binding of the second antibody to the native molecule was

prevented due to epitope masking or steric hindrance. Finally there would be a discrepancy in bFGF measurement between the RIA and the ELISA if a majority of the FGF present in the conditioned medium were aFGF which was cross reacting in the RIA but was not able to bind two antibodies in the RIA.

The heparin-Sepharose affinity chromatography elution profile of BREC conditioned medium shows the presence of 3 peaks of immunoreactivity which were eluted at 0.15M, 0.8M and 2.3M NaCl. The final peak corresponds to the eluting position of rbFGF whereas the second peak is at a salt concentration at which aFGF was eluted by other investigators (Klagsbrun et al., 1987). Three peaks of FGF bioactivity were also seen in the heparin-Sepharose profile of a retinal extract (Courty et al., 1985) and these authors suggested that the first peak was a weakly bound high molecular mass fraction whilst the other two were equivalent to acidic and bFGF. There is considerable variability in the NaCl concentrations at which acidic and bFGF are eluted from heparin-Sepharose by different investigators and this may reflect variations in batches of heparin-Sepharose (Gospodarowicz, 1987). However, if the second peak in our elution profile of BREC is due to aFGF it would represent high concentrations because of the low cross-reactivity of aFGF in the RIA. High concentrations of aFGF in BREC conditioned medium could be responsible for the discrepancy between measurements of bFGF in the RIA and the ELISA. Some of the immunoreactivity present in this peak may be due to bFGF co-eluting at a low salt concentration (Baird et al., 1985b) however recently bovine aortic endothelial cell lysates and conditioned medium have been shown to contain aFGF by heparin-Sepharose chromatography and RIA (McNeil et al., 1989; Speir et al., 1991). We used immunoblotting to clarify this question and aFGF was found to be present in BREC conditioned medium but not at sufficient concentrations to produce the peak which cross-reacted in the bFGF RIA.

If the concentrations of bFGF measured by the ELISA are correct and a substantial proportion of the bFGF immunoreactivity present in BREC conditioned medium represents degraded molecules these may not be bioactive. The

results of the bioassay show that BREC conditioned medium is capable of stimulating DNA synthesis in these cells however other growth factors released from the cells may contribute to or be responsible for all of this activity. The concentrations of bFGF measured in the ELISA, (0.024 pmol/10^6 cells), represents a concentration in the medium of 12pM and this is still above the ED_{50} concentration for the stimulation of BREC proliferation as measured by the methyl-^3H thymidine incorporation assay and Coomassie blue staining.

The results of the experiments measuring bFGF release from BREC with time and in response to cell damage and lysis show growth factor release into serum-free medium associated with a decrease in the number of cells in the monolayer. Its release is also associated with a rise in the quantity of cells and cell debris material in the medium and with release of the enzyme LDH, which provides a marker of cell lysis (Moss & Butterworth, 1974). The quantity of bFGF released was variable and was dependent upon the batch of cells used. The quantity of cells and debris in the medium increased more rapidly than the decrease in monolayer cell number. This could be due to either proliferation of cells in the monolayer or to the degradation of released cells. The presence of cellular debris in the medium indicated that there was break up of the cells.

In serum-containing medium, bFGF release can also be linked to parameters of cell damage and lysis. Although there is an initial increase in monolayer cell number and a less marked linear increase in cell debris due to less cell break up, LDH release was similar to that in serum-free medium indicating that cell lysis did occur. The fact that LDH concentration increases linearly to day 6 in both serum-free and serum-containing medium whereas bFGF release forms a plateau after day 4, could be due to either degradation of bFGF leaving it unable to bind to the antibody in the RIA or to release of all the available bFGF with no further synthesis.

In order to provide more evidence for the relationship between cell damage, lysis and bFGF release, cell growth conditions were chosen so as to produce minimum (sub-

confluent cells) and maximum (lipopolysaccharide treated cells) cell damage. Endotoxin-mediated cell damage had been used previously to investigate the release of growth promoting activity from bovine aortic endothelial cells (Gajdusek & Carbon, 1989). Release of bFGF into both serum-free and serum-containing medium was greatest when the cells were treated with lipopolysaccharide, which also produced the largest decrease in monolayer cell number and increase in cell debris and LDH concentration in the medium. A lower but significant release of bFGF was also seen when serum-free or serum-containing DMEM was conditioned by sub-confluent BREC monolayers. Although there were few cell-debris particles released into the medium indicating little cell break up, LDH was released in quantities approaching those seen from confluent monolayers, showing that cell lysis had occurred and providing an explanation for bFGF release. Due to the difficulties in preparing ECM, which was compounded by the break up of the cell monolayer seen in these experiments, it was not possible to quantify bFGF in the matrix and assess its contribution to the bFGF released into the conditioned medium. The limited data we have produced on bFGF levels extracted from the ECM suggest that the amount present in the matrix is low compared to the quantity within the cell and that present in the conditioned medium. McNeil et al., (1989) have provided evidence that bFGF released from wounded bovine aortic endothelial cell monolayers into the culture medium originates from within the cell and not from the ECM.

At the end of each experiment the flasks to which fresh serum-free medium had been added daily showed more disruption to the monolayer than the flasks used for measuring serum-free cumulative growth factor release. The cumulative cell and debris count in the serum-free medium and the LDH levels were the same in both sets of flasks, as was IGF-1 release until levels reached a plateau. The plateau of IGF-1 release could be due to a cessation of IGF-1 release in these flasks or to the degradation of IGF-1 by proteases released from lysed cells. We have shown IGF-1 release into serum-free medium conditioned by BREC. However, there was no release into serum-containing medium or when the cells were damaged

by the addition of lipopolysaccharide. These results suggest that IGF-1 is not released from damaged BREC. IGF-1 possesses a signal peptide and can be conventionally secreted from cells (Clemmons & Shaw, 1983). It is likely that IGF-1 is secreted into serum-free medium. In serum-containing medium there may be negative feedback on release owing to the presence of IGF-1 in serum. The levels of IGF-1 measured in serum-free medium conditioned by BREC are much higher than those seen in human dermal fibroblasts, 6.5ng/10^5 cells compared to 0.038ng/10^5 cells (Clemmons & Shaw, 1983) however the maximum concentration of IGF-1 reached in our cultures, 2.56nM, is of the same order as that measured in the medium of a human carcinosarcoma cell line of 1.3nM (Van Wyk, 1984). The high levels of IGF-1 measured by us may be due to interference of IGF-1 binding proteins in the RIA. Acid/ethanol stripping successfully removes binding proteins from human serum but may not do so from bovine serum (Mesiano et al., 1988), therefore if BREC secrete IGF-1 binding proteins, as has been shown for other endothelial cells (Bar et al., 1987), these may not be removed and thus interfere in the RIA. IGF-1 may contribute to the retinal endothelial cell proliferation seen in proliferative diabetic retinopathy however we found no effect of IGF-1 on BREC proliferation and no augmentation of the response to bFGF.

Our results show that bFGF is released from damaged BREC however this does not rule out the contribution of bFGF release from the ECM or from cells by an unconventional mechanism. Basic FGF may be released through transient disruptions to the plasma membrane perhaps during cell locomotion (McNeil et al., 1989). There have also been three studies demonstrating alternative methods of release for proteins which do not possess signal peptide sequences. The export of a lactose-binding muscle lectin from mouse C2 myoblasts has been demonstrated (Cooper & Barondes, 1990). The lectin becomes concentrated in evaginations of the plasma membrane which are pinched off to form labile lectin-rich extracellular vesicles. The authors suggest that this is a mechanism for the translocation of the lectin into the ECM and there has been speculation that bFGF could also be

released by this process (D'Amore, 1990). The second possible mechanism is an energy dependent process involving a plasma membrane protein (P-glycoprotein) which has been shown to transport a protein, whose precursors do not contain a signal sequence, out of yeast cells. Similar P-glycoproteins are found in mammalian cells and are responsible for multidrug resistance. A role for these proteins has been proposed in the efflux from cells of interleukin 1 which is not translated with a signal sequence (McGrath & Varshavsky, 1989). Finally another mechanism for the release of interleukin 1ß has been described (Rubartelli et al., 1990). This involves the sequestration of cytoplasmic interleukin-1ß into vesicles allowing subsequent release. In addition to the lack of a signal peptide sequence, bFGF shares significant sequence homology with interleukin 1ß and may be released by one of the above mechanisms.

Northern blotting of human astrocytoma and BREC extracts revealed the presence of mRNA transcripts in these cells. This confirms the data of others (Murphy et al., 1988a; Bensaid et al., 1989a). However, it is the first demonstration of the ability to detect bFGF mRNA transcripts in BREC without using prior mRNA selection from total RNA. The influence of added factors on the levels of bFGF mRNA was not able to be determined due in part to degradation of mRNA transcripts by RNAases.

Chapter 7. THE EFFECT OF GLUCOSE AND LOW OXYGEN TENSIONS ON BREC PROLIFERATION AND RELEASE OF bFGF

7.1 Introduction

The blood glucose level is elevated in diabetes even with appropriate treatment and the possible involvement of hyperglycaemia in the development of diabetic retinopathy has been discussed in Section 1.1.4. The effects of increased glucose concentrations on endothelial cells in culture has been reviewed in Section 1.4.2. and these studies have shown delayed cell replication (Lorenzi et al., 1985), increased synthesis of basement membrane components (Cagliero et al., 1988) and activation of PKC (Lee et al., 1989). There is however no data available on the effect of elevated glucose levels on growth factor release from endothelial cells in culture or on their response to added growth factors. Increased production of bFGF by retinal endothelial cells at the elevated glucose levels seen in diabetes could mean more growth factor within the cells or in the ECM being available for release following cell damage, ECM degradation or by a non-conventional release mechanism (see Section 6.7). This could disturb the balance between positive and negative influences on retinal endothelial cell growth (Pardee, 1987). Increased proliferation of retinal endothelial cells grown in high glucose to added rbFGF would indicate an increased ability of these cells to respond to bFGF released from other retinal cells or by an autocrine mechanism.

We therefore examined the release of bFGF into the culture medium of BREC grown at different glucose concentrations for 8 days compared to cells grown at an equivalent range of mannitol concentrations to control for osmotic effects. We also measured IGF-1 release in order to compare bFGF release with that of a secreted growth factor. The proliferative response of BREC grown at different glucose and mannitol concentrations to bFGF was also examined. For both experiments the range of glucose concentrations used was chosen to encompass those seen in the serum of non-diabetics and diabetic patients with both good and poor diabetic

control. The range was extended to 40mM in order to clarify any dose response effects.

The development of large areas of ischaemic retina is the prelude to the onset of retinal angiogenesis in proliferative diabetic retinopathy. Local hypoxia may therefore be the precipitating factor which instigates endothelial cell invasion, migration and proliferation. Hypoxia may bring about the release of angiogenic growth factors. Alternatively, it could increase the proliferative response of retinal endothelial cells to growth factors present in the retina.

We have grown BREC under reduced oxygen tension in the culture medium. When culture medium is allowed to equilibrate with 95% air:5% CO_2 under standard culture conditions the pO_2 in the medium is around 165 mmHg we have reduced this to 40 mmHg using a mixture of 95% nitrogen:5% CO_2 and investigated its effects on the proliferation of BREC and on their release of bFGF. Although the oxygen tensions used in these experiments were higher than those measured directly above the retina in patients with proliferative diabetic retinopathy which ranged from 14.8 to 31.4 mmHg (Maeda et al., 1990) they may provide evidence for a trend which could be continued at lower oxygen concentrations.

7.2 The effect of glucose on bFGF release from BREC monolayers

7.2.1 Method

Preparation of culture medium supplemented with glucose and mannitol

The formulation of DMEM used for growing BREC contained 5.6 mM glucose. When diluted with 10% v/v tryptose phosphate broth and 10% v/v PDS the glucose concentration was 5.2 as measured by a blood glucose analyser (2300 STAT, Clandon Scientific, Aldershot, U.K.). The glucose concentration of the tryptose phosphate broth was 7.0mM glucose and the PDS contained no detectable glucose. Using this as a baseline

medium appropriate amounts of D-glucose were added to produce solutions containing 10, 20 and 40 mM glucose. Mannitol was added to solutions of baseline medium to give 10, 20 and 40 mM combined glucose and mannitol solutions. All medium was filtered through an 0.2 μ filter before use. The final glucose concentrations were checked with a glucose analyser and found to be within 7% of the calculated values.

Growing BREC in a range of glucose and mannitol concentrations

A 75cm^2 flask of sub-confluent primary culture BREC was trypsinized and the cells divided equally into 8 x 25cm^2 flasks and allowed to attach overnight in DMEM containing 10% v/v PDS. The medium was removed and the monolayer washed once with HBSS after which medium containing one of the four glucose concentrations (5, 10, 20 and 40 mM) or control mannitol concentrations (5mM glucose + 0, 5, 15 and 35 mM mannitol) was added to each flask. The cells were grown for 4 days, at which time they were still subconfluent and were trypsinized. The number of cells in each flask was counted with a Coulter counter.

Growth factor release experiments

The cells grown at each glucose or mannitol concentration were seeded at 1.5 x 10^5 cells per well into the four corner wells of two 6-well cell culture plates. The corner wells were used to prevent the possibility of unequal rates of evaporation differentially affecting the growth of cells in these and in the central pair of wells. The cells were grown to confluence in medium containing the appropriate glucose or mannitol concentration with one change of medium after 48hrs. After 4 days all the cell monolayers were confluent. The medium was removed, the monolayer washed once with HBSS and 1ml of serum-free DMEM containing 0.01% CHAPS was added to each well. The medium was conditioned by the cells for 48hrs and was then removed and the volume noted. After centrifugation at 670g to remove cells and debris the medium

was frozen at -20°C prior to growth factor assay. The cells remaining in the wells were trypsinized, a single cell suspension produced and counted with a Coulter counter.

Radioimmunoassay for bFGF and IGF-1 was carried out on each sample of medium and the results calculated as growth factor concentration in fmol per 10^5 cells in the monolayer. The experiments were carried out on four separate batches of cells.

7.2.2 Results

The results are shown in Table 7.1. Both sets of data were compared by one-way ANOVA. There was no significant difference in the release of bFGF from cells grown at each of the glucose or mannitol concentrations ($p>0.05$). Similarly there was no significant difference in the release of IGF-1 from cells grown in different concentrations of glucose or mannitol ($p>0.05$).

Glucose conc. (nM)	Mannitol conc. (nM)	bFGF conc. (fmol/10^5 cells)	IGF-1 conc. (fmol/10^5 cells)
5	0	34.9 ± 7.8	57.1 ± 7.7
10	0	36.2 ± 6.1	47.1 ± 8.6
20	0	33.3 ± 7.3	43.8 ± 6.8
40	0	32.1 ± 3.9	42.3 ± 4.3
5	0	31.8 ± 5.0	48.0 ± 10.8
5	5	31.8 ± 6.1	53.7 ± 16.0
5	15	32.5 ± 9.9	54.4 ± 15.3
5	35	27.0 ± 4.3	51.9 ± 10.1

Table 7.1 Release of bFGF and IGF-1 from BREC monolayers grown in different glucose and mannitol concentrations. The results are the mean ± SEM of data from 4 separate batches of cells.

7.3 The effect of glucose on the proliferation of BREC and their response to bFGF

7.3.1 Method

Optimization of the mitomycin concentration

Mitomycin-C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan), an antitumour antibiotic which inhibits cell division by intefering with DNA synthesis, was assessed for its ability to inhibit methyl-^{3}H thymidine incorporation into BREC. It was used in the experiment to rule out any effects of glucose and mannitol on the non-specific incorporation of methyl-^{3}H thymidine. The dose used was determined by seeding BREC at 5000 cells per well in a 24-well plate. After overnight attachment the cells were washed with HBSS and mitomycin-C at 25, 50, 100 and 200 $\mu g ml^{-1}$ in HBSS was added, one concentration per row of wells, for 30mins at 37°C. The cells were washed with HBSS and methyl-^{3}H thymidine incorporation was measured over 48hrs. Alternatively, BREC were seeded at 2500, 5000, 10,000 and 20,000 cells per well in each row of a 24-well plate and mitomycin-C (50 $\mu g ml^{-1}$) was added as above followed by the measurement of methyl-^{3}H thymidine incorporation. The results are shown in Figure 7.1 (a and b). The inverse dose response relationship of mitomycin concentration to thymidine incorporation indicated that the 3 lower doses of mitomycin had not completely inhibited DNA synthesis. An increase in mitomycin concentration above 200 $\mu g ml^{-1}$ may have reduced incorporation further but it was decided that this dose produced a low enough count so that an effect of treatment on non-specific methyl-^{3}H thymidine incorporation, large enough to have effected the count in the non-mitomycin treated groups, would have been detectable.

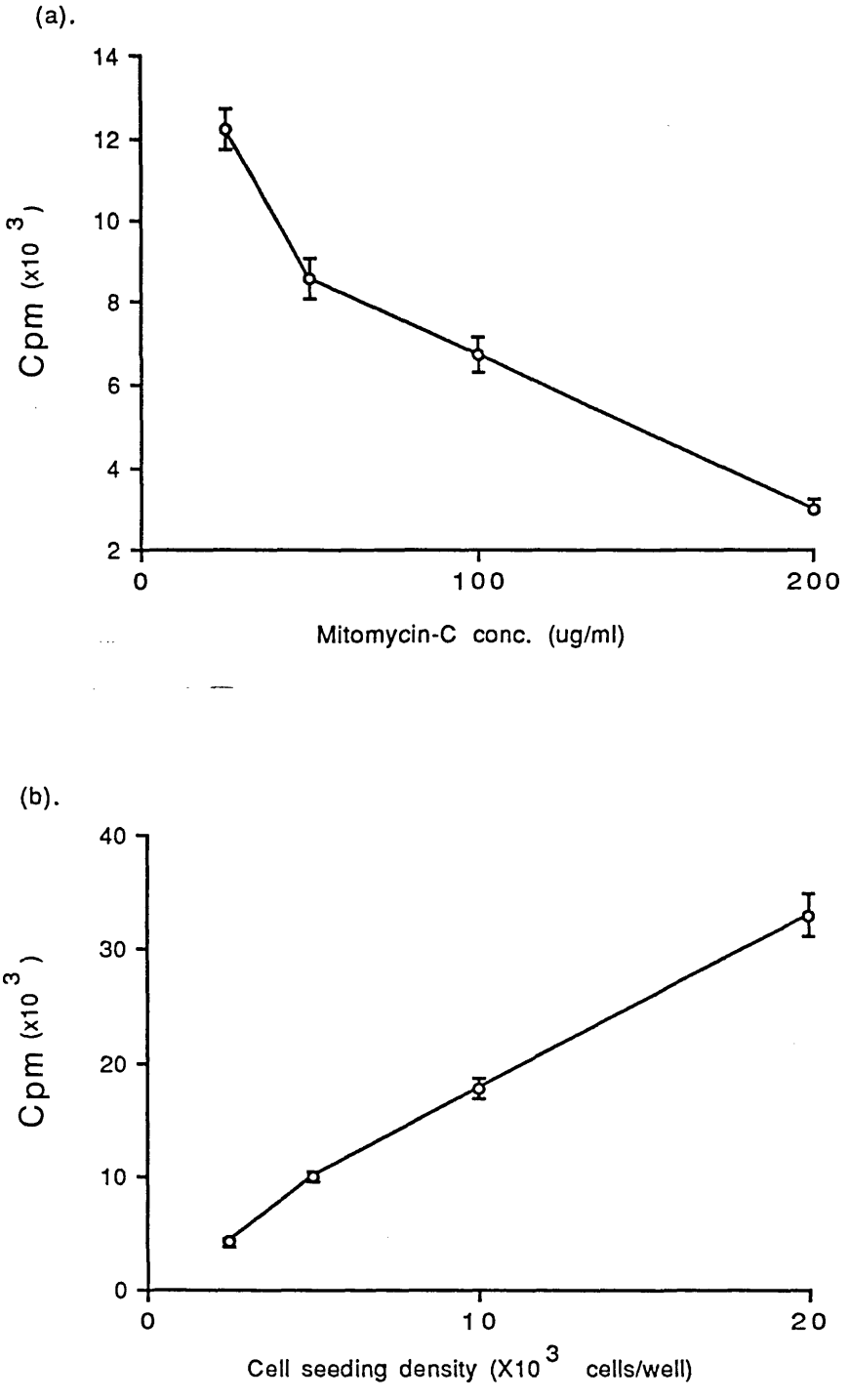


Figure 7.1 Methyl-^{3}H thymidine incorporation into BREC as a function of (a) mitomycin-C concentration and (b) cell seeding density. Each point represents the mean ± s.d. of six replicate wells

Growing cells in medium containing glucose and mannitol

First passage BREC were grown in medium containing a

range of glucose and mannitol concentrations (5-40 mM) as described in 7.2.1 for four days after which they were trypsinized and counted.

Measuring methyl-^{3}H thymidine incorporation

Counted cells were seeded at 5000 cells per well into the wells of two 24-well plates in DMEM containing 10% v/v PDS. The appropriate glucose and glucose/mannitol concentrations were added with one concentration per row. After overnight attachment the cells were washed with HBSS and DMEM containing 0.1% v/v PDS and the appropriate glucose or glucose/mannitol concentration was added. After a further 24 hours the wells were washed and the thymidine incorporartion assay carried out with two wells pre-treated with mitomycin-C (200 $\mu g ml^{-1}$) and two wells treated with bFGF (5 pM) at each hexose concentration.

7.3.2 Results

The results are shown in table 7.2. No significant difference was found between the proliferation of BREC at any of the glucose or glucose/mannitol concentrations when analysed by one-way analysis of variance ($p>0.05$). There was also no significant difference between the cells grown at different hexose concentrations in their proliferative response to bFGF (range 8.5-37.5% increase over no treatment, $p>0.05$) or their methyl-^{3}H thymidine incorporation following treatment with mitomycin-C (range 18.2-26.0% of untreated cells, $p>0.05$) indicating no effect of treatment on non-specific methyl-^{3}H thymidine incorporation.

Added hexose	Control (cpm)	Mitomycin treated (cpm)	%C	Basic FGF treated (cpm)	%C
G5	1695 ± 287	385 ± 117	22.7	1839 ± 232	108.5
G10	1820 ± 164	332 ± 62	18.2	1988 ± 217	109.2
G20	1893 ± 226	427 ± 114	22.6	2603 ± 375	137.5
G40	2142 ± 313	513 ± 146	23.9	2666 ± 425	124.5
G5	1697 ± 220	364 ± 68	21.4	2180 ± 231	128.5
G5+ M5	1715 ± 198	425 ± 102	24.8	2242 ± 177	130.7
G5+ M15	2060 ± 145	535 ± 156	26.0	2497 ± 245	121.2
G5+ M35	2099 ± 292	469 ± 75	22.3	2640 ± 311	125.8

Table 7.2 The proliferative response of BREC grown in medium containing different glucose or glucose/mannitol concentrations, either basal, treated with mitomycin-C or stimulated with bFGF. Results are the mean ± SEM from 5 separate experiments and are also shown as a percentage of the control (%C) for mitomycin and bFGF treated cells.

7.4 The effect of hypoxia on BREC proliferation and release of bFGF

7.4.1 Method of growing BREC under reduced oxygen tensions

The partial pressure of oxygen in the cell culture medium

was reduced by bubbling a stream of nitrogen through serum-free DMEM containing 10% v/v tryptose phosphate broth for 5 minutes. A sample was taken using a syringe and the oxygen partial pressure measured using an ABL 300 blood gas analyser (Radiometer, Copenhagen, Denmark). Nitrogen displaced oxygen from the medium and reduced the oxygen partial pressure from 168 ± 9 mmHg to 44 ± 15 mmHg (mean ± s.d.; n=5) The medium was kept under a stream of nitrogen until used. Plasma derived serum was added to a concentration of 10% v/v and after mixing a further sample was taken for blood gas analysis.

7.4.2 Proliferation of BREC in response to reduced pO_2

Method—

First or second passage endothelial cells were trypsinized and a single cell suspension produced. The cells were counted and seeded at 30,000 cells per flask in 9 x 25 cm^2 tissue culture flasks. After overnight attachment the medium was removed. Three flasks were trypsinized and counted to provide baseline cell number. Fresh DMEM containing 10% PDS was added to 3 of the remaining flasks (high O_2 flasks) and medium in which the oxygen tension had been reduced as described above was added to the other 3 flasks (low O_2 flasks). A mixture of 95% v/v air and 5% v/v CO_2 (B.O.C Medical Gases, Brentford, U.K.) was used to flush the high O_2 flasks and a mixture of 95% N_2 and 5% CO_2 delivered via an anaesthetic flow meter was blown into the low O_2 flasks to displace the existing gas mixture. The flasks were sealed and incubated for 48hrs at 37°C.

After incubation a sample of medium was removed and the oxygen partial pressure, the CO_2 partial pressure and the pH were measured. The rest of the medium was removed, the monolayer trypsinized and the cells counted. The experiment was carried out on 5 separate batches of cells.

Results

All batches of cells responded to the low oxygen tension in the medium with increased cell proliferation shown by the cell count at 48hrs (see Fig. 7.2). This data can be divided into two groups depending on whether the cells used in the experiment were 2nd or 3rd passage. The initial cell counts were 0.33 ± 0.02 ($x10^5$) (mean ± s.d; n=3) cells per flask for 2nd passage cells and 0.18 ± 0.02 ($x10^5$) cells per flask for 3rd passage BREC indicating that second passage cells attached more readily to the substrate. However both groups showed an increased cell count at 48 hours between cells grown in high and low oxygen concentrations. The increases were from 2.5 - 3.1 ($x10^5$) cells per flask in 2nd passage cells, an increase of 24% and from 0.3 - 0.6 ($x10^5$) in 3rd passage cells, an increase of 10%. The cell counts from the high and low oxygen flasks were compared using the paired Student t-test and were significantly different (p=0.008). The oxygen tensions at the start of the experiment were 165 mmHg and 45 ± 16 mmHg (mean ± s.d.) in the high and low O_2 flasks and at the end of the experiment were 165 ± 6 mmHg and 96 ± 22 mmHg.

7.4.3 Release of bFGF from cells grown under reduced oxygen tension

Method

First passage BREC were trypsinized and counted with a Coulter counter. They were seeded at 30,000 cells per flask in 9 x 25cm^2 flasks. After 24 hours 3 flasks were trypsinized and the cells counted. In the remaining flasks the medium was changed to give 3 low O_2 flasks and 3 high O_2 flasks as described in Section 7.4.2 above. The cells were incubated at 37°C for 48hrs and the medium removed. The oxygen partial pressure was measured in the medium with a blood gas analyser. The cells were washed once with HBSS and serum-free DMEM containing 0.01% w/v CHAPS was added to each flask and the cells incubated at 37°C. After 48hrs the medium was

harvested, centrifuged at 670g to remove debris and frozen at -20°C prior to growth factor assays.

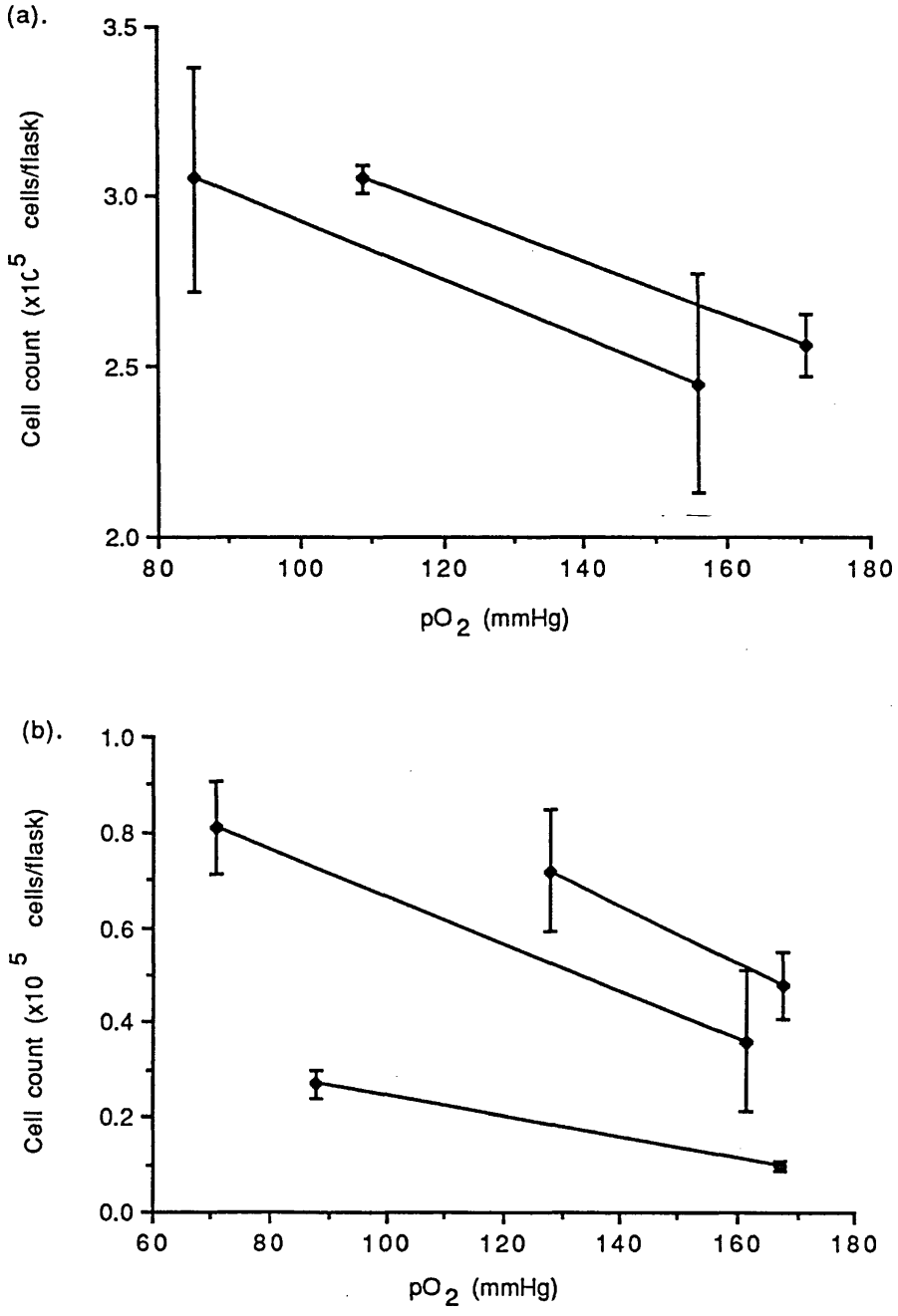


Fig. 7.2 Bovine REC number after 48 hrs as a function of the partial pressure of oxygen in the medium using (a). 2nd passage cells and (b). 3rd passage cells. Each point represents the mean ± SEM of triplicate flasks.

The cell monolayers were trypsinized, a single cell

suspension produced and the cells counted. Radioimmunoassays for bFGF and IGF-1 were carried out on the conditioined medium. The experiment was carried out on 4 separate batches of endothelial cells.

Results

The cell counts at the start of the experiments were 3.9 ± 0.3, 4.6 ± 0.8, 4.4 ± 0.6 and 4.1 ± 0.3 ($x10^4$ cells/flask) (mean ± s.d.) for the four diferent batches of cells. The oxygen tension in the medium before addition to the flasks was 164 mmHg (high O_2) and 39 mmHg (low O_2). After 48hrs the oxygen tensions were 156 ± 6 mmHg (high O_2) and 73 ± 7 mmHg (low O_2) (mean ± s.d of four flasks, one flask from each batch of cells). The cell number in the flasks of each batch and the bFGF release are shown in Table 7.3. The IGF-1 concentrations in the medium were below the sensitivity of the RIA.

Batch	Cell count ($x10^5$)		bFGF (fmol/10^5 cells)	
	Low O_2	High O2	Low O2	High O2
1	2.8 ± 0.3	2.7 ± 0.5	19.8 ± 5.1	23.6 ± 2.2
2	2.5 ± 0.2	2.2 ± 0.3	22.6 ± 4.6	21.2 ± 4.3
3	3.3 ± 0.2	2.5 ± 0.6	15.3 ± 2.2	29.7 ± 6.1
4	2.1 ± 0.3	2.4 ± 0.1	26.3 ± 3.0	18.3 ± 0.7
Mean	2.7 ± 0.5	2.5 ± 0.2	21.0 ± 4.6	23.2 ± 4.8

Table 7.3 Cell number and bFGF release from cells grown at two different oxygen tensions. Data are the mean ± s.d. of triplicate flasks

Although there was a slightly increased cell count at the end of the experiment in the flasks subjected to a low oxygen

tension when compared to those under standard culture conditions (high O_2) (2.7 ± 0.5 versus 2.5 ± 0.2 ($x10^5$ cells/ flask; mean ± s.d: n=4), this was not significant. The experiment did not show as large a difference in cell count in the low O_2 compared to the high O_2 flasks as that seen in the previous series (Section 7.4.2). This could be due to the difference in experimental protocol. After 48hrs at different oxygen tensions in this experiment, the cells had 48hrs in serum-free medium at uniform oxygen tensions to produce the conditioned medium. During this time a proportion of the cells were released from the monolayer into the medium which effected the final cell count.

There were differences within the batches in the amount of bFGF released between high and low O_2 flasks however these were not consistent and there was no significant difference in mean bFGF release between high and low O_2 flasks (23.2 ± 4.8 versus 21.0 ± 4.6 fmol/10^5 cells; mean ± s.d; n=4; $p>0.05$).

7.5 Discussion

Bovine retinal endothelial cells in culture did not show any change in DNA synthesis measured over 48 hours when grown in culture medium containing increasing concentrations of glucose or mannitol for 6 days. This does not accord with previous data showing decreased DNA synthesis in human umbilical vein endothelial cells at glucose concentrations of 16.7, 27.8 and 50mM and equimolar mannitol concentrations when compared with control media containing 5.6mM glucose (Porta et al., 1988). The cells in the study of Porta et al., were exposed to the elevated hexose concentrations for a period of 48hrs only and the inhibition of DNA synthesis was attributed to an osmotic effect. A possible reason for the discrepancy between our results is that the cells became adapted to this osmotic effect after an increased length of exposure to raised levels of glucose and mannitol and this is supported by the finding that there was no decrease in cell number after 96 hours exposure to increased mannitol levels (Porta et al., 1988). A significant decrease in cell counts

after 96 hours exposure to 27.8 and 50mM glucose concentrations was seen by Porta et al., (1988) and these findings confirmed those of Lorenzi et al., (1985) who showed decreased growth of human umbilical vein endothelial cells after 6-16 days exposure to 20mM glucose compared to cells grown at 5mM glucose. The effects of glucose on human umbilical vein endothelial cells was attributed to a prolongation of the cell cycle (Lorenzi et al., 1987) although a decrease in incorporation of the thymidine analogue bromodeoxyuridine was not seen in these studies, in agreement with our findings. A difference in cell type could also be involved in the contrasting results seen between us and Porta et al., (1988) and this possibility is strengthened by data showing no effect of 4 days exposure to 27.8 mM glucose on BREC number (Porta et al., 1990).

PKC has been implicated in the signal transduction of receptors involved in the mitogenic and chemotactic response of bovine cerebral cortex endothelial cells to bFGF (Daviet et al., 1990) and in the mitogenic response of Swiss 3T3 cells to bFGF (Tsuda et al., 1985). Also, the proportion of the active, membranous pool of PKC was increased by 100% in BREC grown in medium containing 22.2mM glucose for 5 days compared to cells grown in a glucose concentration of 5.6mM (Lee et al., 1989). Our results show no effect of elevated concentrations of glucose or mannitol on the proliferative response of BREC to rbFGF indicating that exposure to elevated glucose does not increase the sensitivity of BREC to the growth stimulatory effects of this factor. Therefore if PKC is activated in our cells by increased glucose this does not alter the proliferative response of these cells to bFGF.

Exposure of endothelial cells to increased concentrations of glucose has been shown to increase the synthesis of basement membrane components and this could alter their proliferative reponse to growth factors (Cagliero et al., 1988). However we showed no effect of the substrate on the proliferative response of BREC to bFGF (see Section 4.2.3) and this would agree with our findings of no effect of increased glucose on the proliferation of BREC.

There was no increased release of bFGF or IGF-1 from BREC

cultured in elevated glucose and mannitol concentrations for 8 days. Lorenzi et al., (1985) report a significantly increased amount of cell death in human umbilical vein endothelial cells grown at 20mM glucose, but not mannitol, compared to those grown at 5mM glucose. Increased cell death was measured as an increase in the fraction of LDH in the medium over LDH in the cell monolayer. We have shown an increased release of bFGF from confluent BREC monolayers with time and associated this with cell damage and an increase in LDH in the medium (see Section 6.5). The release of bFGF from BREC might be expected in cultures grown in elevated glucose concentrations if there was increased cell death and LDH release. However, the increase in LDH in the medium of control cultures compared to those grown at elevated glucose concentrations calculated from the data of Lorenzi et al., (1985) was 3.2 IU per litre. This value compares with a daily increase of LDH of 105 IU per litre associated with measurable increases in bFGF release in our study so differences in bFGF release due to increased cell death and lysis at elevated glucose concentrations would be very small and within the variability of bFGF measurement in our assay.

The increased proliferation of BREC measured by us when cells were grown at decreased oxygen concentrations in the culture medium are in agreement with the results of Rosen et al., (1989) who showed increased proliferation of retinal endothelial cells and pericytes grown at reduced oxygen concentrations on a gas permeable substrate and decreased proliferation of these cells at elevated oxygen levels. A possible mechanism for this increased proliferation could be a reduction in the levels of free radicals. Oxygen is toxic for cells and there may be toxic effects even at atmospheric oxygen concentrations (21% v/v O_2) (Halliwell & Gutteridge, 1989). The main mechanism for this toxicity is via the generation of free radicals. These factors damage lipids in the cell membrane and may interfere with the normal growth response of cells in culture to growth promoting factors in the culture medium. Alternatively cells could be damaged by low oxygen tensions and release bFGF which would stimulate increased proliferation in the undamaged cells promoting an

overall increase in cell number. However, oxygen tensions in the culture medium as low as 12-14 mmHg did not damage bovine adrenal microvascular endothelial cells (Ogawa et al., 1990 and extracellular pO_2 values below 5 mmHg are required to significantly impair the cytochrome oxidase activity of the mitochondrial respiratory chain (De Groot and Littauer, 1989), so endothelial cell damage at the oxygen tensions seen in our study is unlikely.

Shreeniwas et al., (1991) showed a decrease in the proliferation of bovine adrenal capillary and aortic endothelial cells at oxygen tensions below 47 mmHg. They also showed that these cells were able to respond to bFGF by increased proliferation in a dose dependant manner. The latter response was mediated by a 3 fold increase in the number of high affinity bFGF receptors with no change in binding affinity however there was also a decrease in the amount of bFGF extracted from the cells. We have shown no difference in the amount of bFGF released into the culture medium by cells grown at low oxygen tensions. If bFGF receptor number also increased in our cells, this would provide a mechanism for the increased cell proliferation.

We have demonstrated increased proliferation of BREC in response to reduced oxygen concentrations in the culture medium however, the oxygen tensions achieved were equivalent to those seen in normal vessels and not relevant to the oxygen tensions in ischaemic retina. If there is a further increase in cell proliferation at even lower oxygen tensions then this may be responsible for instigating or contributing to new vessel growth in the ischaemic retina although the mechanisms involved are not yet understood.

CHAPTER 8. FINAL DISCUSSION AND FUTURE DIRECTIONS

Proliferative diabetic retinopathy is the consequence of a sequential series of pathological changes to the retina and the retinal vessels brought about by the metabolic disturbances of diabetes. The common finding prior to the onset of retinal neovascularization is capillary occlusion and the development of areas of ischaemic retina. The process of angiogenesis then commences with the breakdown of the basement membrane and extracellular matrix surrounding small venules, the migration of venular endothelial cells into the stroma followed by endothelial cell proliferation and lumen formation. This process is undoubtedly a homeostatic mechanism by the retina to reoxygenate the areas of hypoxia and is also seen in other retinal pathologies where retinal ischaemia is evident. The new vessel growth is however not under the same degree of control as it is in other cases of angiogenesis, for example in embryological vascular development and in wound healing.

How oxygen deficiency is translated into the stimulus for the endothelial cell changes seen during angiogenesis is unknown. It is likely however that some of the metabolic alterations of diabetes and the retinal changes produced during the development of diabetic retinopathy predispose the retina to neovascularization. In normal tissue endothelial cell proliferation and blood vessel growth are very tightly controlled and inhibitory influences are dominant. In the diabetic retina a series of events is likely to be critical in the onset of neovascularization. First there is the loss of inhibitory influences. There is pericyte dropout from the retinal capillaries early in the development of diabetic retinopathy and retinal pericytes in culture have been shown to exert an inhibitory influence on endothelial cell proliferation (Orlidge & D'Amore, 1987). In addition there are changes to the capillary basement membrane in diabetic retinopathy which may effect endothelial cell attachment. A change in the response of capillary endothelial cells to bFGF from one of differentiation to one of growth has been associated with a decrease in the adhesion of these cells to

the ECM (Ingber & Folkman, 1989). Secondly there is the influence of a circulating factor or factors acting either directly on the luminal side of the endothelium or passing from the circulation via the leaky blood retinal barrier into the retina and the vitreous and finally there is the elaboration of a locally released growth factor or factors.

In this thesis we have proposed that the local stimulus to retinal neovascularization is bFGF, a potent mitogen for capillary endothelial cells which has been implicated as being involved in all the stages of angiogenesis. Basic FGF lacks a signal peptide for conventional secretion and it is therefore likely that this growth factor has to be released either from the extracellular matrix surrounding the vessels or from within the cells themselves by an unconventional mechanism. We have shown the presence of bFGF and mRNA transcripts required for its synthesis in BREC. We have also shown bFGF associated with the extracellular matrix produced by these cells. The concentrations of bFGF within the cells is high as measured by RIA and the levels in matrix are much lower. Basic FGF levels in endothelial cells in vivo are not thought to be as high as those seen in cultured cells (Speir et al.,1991) and this illustrates one of the difficulties with data obtained from in vitro models.

We have shown that BREC in culture release bFGF into the culture medium when the cell membrane is damaged as shown by the concomittant release of LDH, a large intracellular enzyme. The levels of bFGF released by BREC as measured by RIA were again very high but much lower levels were seen when bFGF was measured with the more specific ELISA. However, even the lower of these two concentrations is sufficient to stimulate the proliferation of BREC in vitro.

Basic FGF could be released from retinal endothelial cells damaged following capillary occlusion and when this became widespread, sufficient quantities of growth factor could be released to instigate the angiogenic process. Alternatively, the release of bFGF from damaged BREC could provide the link between hypoxia and neovascularization. Low oxygen tensions could produce retinal cell damage due to inhibition of oxidative phosphorylation with subsequent

release of bFGF from intracellular stores. The retinal endothelial cells are however unlikely to be damaged by retinal hypoxia. Oxygen tensions as low as 14 mmHg in the culture medium were not toxic to endothelial cells (Ogawa et al., 1990) and in retinal explant cultures, which developed extensive retinal ischaemia, there was no evidence of endothelial cell death, on the contrary these were the cells which proliferated (Forrester et al., 1990a). These observations do not however rule out an increase in transient disruptions to the endothelial cell plasma membrane which could release bFGF. In the explant cultures the surrounding neuronal tissue did however die and these cells could be a source of bFGF. Retinal ganglion cells, neurones and photoreceptors have been shown to contain both acidic and bFGF (Caruelle et al., 1989; Ishigooka et al., 1991). They are metabolically active and utilise large amounts of oxygen. They are also present in the area of the retina with the lowest oxygen tension and could therefore be affected by reductions in the oxygen supply due to capillary closure. Because the photoreceptors recieve oxygen from the choroidal circulation damage to these cells is likely to be secondary to changes to the neuronal cells.

If bFGF is released from retinal cells it is possible that it would pass into the vitreous either across the inner limiting membrane or through the breaches in this tissue produced by the proliferating vessels. The vitreous could act as a sieve, concentrating growth factor at the vitreo-retinal boundary where the new vessels develop. We found no evidence of bFGF in the vitreous of diabetic patients with a history of proliferative diabetic retinopathy. We also measured IGF-1 and showed no difference between patients with and without diabetic retinal disease. However, these results are in contrast to those of other groups and the levels of bFGF found by Sivalingam et al., (1990) were particularly high. It is likely that the inner limiting membrane itself contains heparan sulphate proteoglycan molecules and can bind bFGF and one study has localized this growth factor to the membrane immunocytochemically (Jeanny et al., 1987). Localization to the inner limiting membrane could also promote growth of

retinal vessels along the vitreo-retinal boundary although bFGF would not necassarily be measurable in the vitreous. Retinal detachment and vitreous haemorrhage are the main reasons for vitrectomy amongst diabetic patients and it is difficult to obtain pure vitreous uncontaminated with serum proteins from patients with active proliferative diabetic retinopathy. More studies are required to clarify the types and levels of growth factors present in the vitreous during retinal neovascularization.

More than one growth factor is likely to be involved in the development of proliferative diabetic retinopathy and the interaction of bFGF with ESAF, another growth factor found in the retina, has been proposed (Odedra & Weiss, 1987). Circulating factors are also likely to be important as evidenced by the beneficial effects of pituitary ablation and the increased incidence and severity of proliferative diabetic retinopathy during and post-puberty. We found no evidence for a stimulatory factor for BREC growth in serum from patients with proliferative or pre-proliferative diabetic retinopathy compared to patients with background or treated inactive proliferative diabetic retinopathy and controls. These results however exposed a weakness in our culture system. We have used bovine retinal endothelial cells for reasons of availability however a proliferative factor has been described in the serum of diabetic patients which is probably only active on human endothelial cells. This factor has not been characterized but was not growth hormone or IGF-1 (Petty et al., 1989). There is evidence however that these two growth factors are involved in proliferative diabetic retinopathy. In our culture system IGF-1 did not stimulate BREC proliferation or exert an additive or synergistic effect with bFGF. However, the in vitro growth response of BREC was small and not consistent between bioassays. The influence of the culture conditions used and the sensitivity of the bioassays we have developed must be borne in mind when evaluating this data.

No effect of raised glucose levels was seen on the proliferation of BREC or their release and response to bFGF. Although elevated glucose is likely to play a part in the

development of retinal pathology prior to new vessel growth it is unlikely to be an acute stimulus at the onset of angiogenesis. The results on the effects of glucose obtained using cell culture must be treated with caution because of the brief time over which the cells were exposed to elevated glucose levels compared to the duration of diabetes prior to the onset of retinal vascular complications.

We have shown that lowering the oxygen concentration in the culture medium results in the increased proliferation of BREC. Although the reduction in oxygen concentration in these studies was not great, the lower oxygen tensions seen in ischaemic retina may similarly stimulate endothelial cell proliferation. This effect of low oxygen concentrations on endothelial cell proliferation was not however due to autocrine stimulation of proliferation following increased bFGF release.

The results we have obtained from these studies are in agreement with our hypothesis that there is release of bFGF from retinal cells prior to the onset of capillary growth which alters the balance of growth influences on the endothelial cells and stimulates their proliferation. The model we have used is however limited. We have only investigated bFGF release from BREC and these cells are unlikely to be damaged by retinal ischaemia. Also the measurement of release of bFGF from damaged cells by RIA is insensitive and does not lend itself to investigations of the factors modulating bFGF production. This information is more likely to be obtained from studying mRNA transcript levels in retinal cells and we have shown that these investigations will be possible using relatively small numbers of BREC per treatment group. The relevance of levels of bFGF mRNA in cultured cells compared to those in vivo does however need to be addressed.

The mechanism proposed here for retinal neovascularization may be plausible for the uncontrolled capillary growth seen in proliferative diabetic retinopathy but it is unlikely that angiogenesis during embryonic development or endometrial development could be regulated by such an uncontrolled mechanism, thus more than one regulatory

system for angiogenesis would be required.

There are alternative theories for retinal neovascularization. The first is a variation on the release of bFGF from damaged cells. This proposes that bFGF stored in the extracellular matrix is released by heparinase or heparinitase enzymes (Baird & Ling, 1987) and retinal hypoxia could provide the signal for elaboration of these enzymes. Evidence in support of this idea is that bFGF has been localized in the matrix surrounding retinal vessels (Hanneken et al., 1987) and heparinase enzymes have been demonstrated in a retinal extract (Forrester et al., 1990b). The second theory concerns the potential involvement of macrophages. Although these cells are more commonly associated with wound healing and immune and non-immune inflammation, they are also present in the retina (Savage et al., 1988). In a retinal explant model of proliferative diabetic retinopathy, Forrester et al., (1990a) demonstrated that activated macrophages were associated with cords of endothelial cells which were growing out of the explants following the development of ischaemia in the explant and necrosis of the neuronal cells. Low oxygen tensions have been shown to activate macrophages and stimulate them to release bFGF (Knighton et al., 1983). Release of bFGF from macrophages may therefore provide a controlled means of stimulating angiogenic activity when the oxygen tension is low and inhibiting it when new capillary growth has reoxygenated the tissue.

The experimental investigations carried out in support of this thesis have emphasized the requirement for well-characterized and reproducible methods for the detection and measurement of biological activity. Both the immunoassays and the bioassays used are limited in the information they can provide. The problems associated with using isolated BREC in culture to model the complex pathological process of retinal angiogenesis are even more apparent and whilst the results we have obtained strengthen our hypothesis more work is required to clarify the details of the process. A more sensitive immunoassay is required to be more certain about the presence or absence of bFGF in serum and particularly vitreous

samples. The latter specimens need to be thoroughly characterized with samples from patients with active new vessel growth and no haemorrhage need to be matched with non-diabetic controls. Also, the immunoassays for bFGF need to be standardized so that results between laboratories can be compared. The extension of the use of immunocytochemical techniques on retinal tissue and particularly vitreo-retinal membranes is required. Again there is the difficulty of obtaining specimens with active neovascularzation however sensitive methods of staining these for bFGF should provide evidence as to its location in relation to the developing neovascular fronds. The use of in situ hybridization techniques will also be important in localizing cells which are actively synthesizing bFGF.

The problems involved in relating the results obtained from monocultures of retinal cells requires the further development of co-culture techniques to examine the interaction between cells and further information derived from retinal explants will be important. In this context the problems posed by the different responses of cells from different species means that experiments carried out with human retinal cells are needed to confirm data obtained with animal tissue. Finally the limitations and variations involved in all these techniques and experimental procedures necessitates confirmation of results using more than one method by several groups before a concensus can be reached.

The goal of research into diabetic retinopathy and the stimulus to retinal angiogenesis is the development of rational methods for its prevention and treatment. Several agents have been developed with anti-angiogenic potential including fumagillin and its synthetic congeners (Ingber et al., 1990). The correlation of data from clinical and laboratory investigations of the effects of these agents will help to clarify the mechanisms involved in the development of the disease and bring about further improvements in its treatment.

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Characterization of release of basic fibroblast growth factor from bovine retinal endothelial cells in monolayer cultures

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Release of basic fibroblast growth factor (bFGF) was investigated in bovine retinal endothelial cells (BREC) maintained in monolayer culture. Confluent cells released bFGF into serum-free culture medium or medium containing 5% serum at rates of up to 105.2 and 61.3 pM/day respectively. bFGF release coincided with a decrease in monolayer cell number and increases in lactate dehydrogenase (LDH) concentration and cells and cell-debris particles in the medium, which suggested that cell damage and lysis were responsible for growth-factor release. Maximum bFGF release at 24 h (230 ± 10 pM) occurred when the cells were treated with lipopolysaccharide (10 μg/ml), which also produced the greatest changes in parameters of cell damage. Sub-confluent cells showed little overt damage at 24 h, but released bFGF (78 ± 20 pM) along with LDH, indicating that some cell lysis had occurred. Insulin-like growth factor 1 (IGF-1) was also released into serum-free culture medium at a rate of 0.34 nM/day, but not into medium containing serum or when the cells were treated with lipopolysaccharide. This implies that the mechanism of IGF-1 release is different from that of bFGF and is not related to cell damage. Culture medium conditioned by BREC stimulated the proliferation of these cells, as measured by an increase in their incorporation of [*methyl*-^{3}H]thymidine from 7550 ± 479 to 10467 ± 924 d.p.m. These results demonstrate that bFGF is released from damaged BREC and that medium conditioned by these cells can stimulate retinal-endothelial-cell proliferation. This strengthens the case for an involvement of this growth factor in retinal neovascularization.

INTRODUCTION

Basic fibroblast growth factor (bFGF) is a polypeptide originally isolated from bovine pituitary and brain [1,2]. It is one member of a family of growth factors characterized by their strong affinity for heparin, which includes acidic fibroblast growth factor [3] and some tumour-cell-derived factors [4,5]. It was originally isolated independently from many cells and tissues and was given several names, e.g. eye-derived growth factor I [6], heparin-binding growth factor β [7] and macrophage-derived growth factor [8], all of which have been shown to be homologous to bFGF [9].

Retinal extracts have been shown to contain bFGF [10], and purified bFGF is a potent mitogen for bovine retinal endothelial cells (BREC) [11]. bFGF is also angiogenic in several models [12,13]. Considerable interest has therefore developed as to its possible role in proliferative diabetic retinopathy. This condition is characterized by the growth of abnormal new blood vessels arising from existing retinal vessels [14].

bFGF is present in the extracellular matrix of cells, where it is bound to heparan sulphate proteoglycans [15,16], and early reports suggested that it was not present in media conditioned by bovine aortic and corneal endothelial cells, although it could be extracted from these cells and from the matrix [17,18]. When the gene for bFGF was cloned [19], it was realized that the transcribed sequence did not contain a classical signal-peptide region allowing secretion from cells, and that in most tissues mRNA levels were very low, suggesting little bFGF production [20].

The release of bFGF from extracellular matrix or from cells is required if it is to be implicated in either physiological or pathological angiogenesis. Two theories have been advanced, the first that extracellular matrix acts as a pool of bFGF which can be released in response to heparinitase enzymes [15,21,22] and the second that cell damage could cause the release of this growth factor from within the cell. Recently, injury-mediated release of bFGF has been demonstrated from bovine aortic endothelial cells [23], and bFGF has been shown to be present in medium conditioned by BREC [24], although it was not clear how release from these cells was mediated.

The aims of this study were (1) to examine the pattern of bFGF release from BREC with time, by using a radioimmunoassay, (2) to relate this release to parameters of cell damage and lysis, (3) to characterize biochemically the bFGF released, and (4) to measure its bioactivity with [*methyl*-^{3}H]thymidine uptake as a marker of cell proliferation.

MATERIALS AND METHODS

Cell culture

BREC were isolated and cultured as previously described [25]. Retinas were dissected from bovine eyes, homogenized in Minimum Essential Medium (Eagle's), filtered through an 85 μm-pore-size filter (Henry Simon, Stockport, U.K.), and the blood vessels trapped on the filter were digested in a 1 mg/ml collagenase–Dispase (Boehringer Mannheim, Mannheim, Germany) solution for 90 min at 37 °C. Vessel fragments were plated on to a fibronectin substrate (New York Blood Centre, New York, NY, U.S.A.) in 75 cm^2 tissue-culture flasks. Culture medium was 8% Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% tryptose phosphate broth and 10% plasma-derived serum (DMEM + 10% PDS) [26]. All culture media were purchased from Gibco, Paisley, Scotland, U.K., and tissue-culture plastics were from Becton Dickinson, Oxford, U.K. Cells grew to confluence in 5 days, when they were passaged by trypsin treatment. Experiments were carried out on first- or second-subculture cells.

Abbreviations used: bFGF, basic fibroblast growth factor; rbFGF, recombinant bFGF; BREC, bovine retinal endothelial cells; IGF-1, insulin-like growth factor 1; DMEM, Dulbecco's Modified Eagle's Medium; HBSS, Hanks Balanced Salt Solution; PDS, plasma-derived serum; LDH, lactate dehydrogenase.

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The cells were characterized by their cobblestone morphology and by uniform staining for Factor VIII-related antigen.

Growth-factor release from BREC monolayers

Subconfluent BREC were treated with trypsin (0.25%, w/v) (ICN Flow, Rickmansworth, Herts., U.K.), and a single-cell suspension was produced by passing cell clumps through a 21-gauge needle. An equal volume of this suspension was then added to each of 24 25 cm^2 tissue-culture flasks previously treated with fibronectin, and the cells were grown in DMEM + 10% PDS. At confluence the medium was removed and the monolayer washed with Hanks Balanced Salt Solution (HBSS). Then 2 ml of serum-free DMEM containing 0.01% (w/v) CHAPS [27] was added to each flask, and the flasks were sealed and incubated at 37 °C; 21 flasks were used for measuring cumulative growth-factor release, and 3 for measuring daily release from the same monolayer, and subsequent results and discussion will refer to either serum-free cumulative or daily release. Photographs were taken of the cells every day (Fig. 1). A further series of experiments was carried out on 21 25 cm^2 flasks containing cells prepared identically with those above. At confluence the medium was removed, the monolayer washed with HBSS and 2 ml of DMEM containing 5% (v/v) human serum (North London Blood Transfusion Centre, London N.W.9, U.K.) was added to each flask, and the flasks were sealed and incubated at 37 °C. These flasks are referred to as serum-containing cumulative-release flasks. At zero time and every day for 6 days, three of the serum-free cumulative-release flasks, three of the serum-containing cumulative-release flasks and the three flasks used for measuring daily release had their medium removed and centrifuged at 670 *g* for 10 min. The medium from each flask was divided into four 0.5 ml portions, and these were stored frozen at −20 °C before growth-factor assays. Samples for growth-factor bioassay were prepared under sterile conditions by using medium removed from the serum-free cumulative-release flasks. The remaining pellet of debris and cells was resuspended in 0.75 ml of HBSS and counted with a Coulter Counter (Coulter Electronics, Luton, U.K.). A 2 ml portion of fresh serum-free DMEM + 0.01% CHAPS was added to each of the three flasks used for measuring daily release. Medium and debris were removed from these flasks each day and fresh serum-free DMEM + 0.01% CHAPS was added. The cell monolayer in the three serum-free and serum-containing cumulative-release flasks was washed with HBSS and treated with 1 ml of 0.25% trypsin until all cells had detached. The cells were resuspended in 1 ml of HBSS and counted.

Growth-factor release from lipopolysaccharide-treated and sub-confluent BREC monolayers

Cell growth conditions were chosen in order to achieve minimum (sub-confluent growing cells) and maximum (addition of lipopolysaccharide) cell damage. BREC were plated into 25 cm^2 flasks at either high density (600000 cells per flask) or low density (200000 cells per flask). The cells in the flasks seeded at high density were grown to confluence, whereas those seeded at low density were allowed to attach overnight at 37 °C (sub-confluent flasks). Medium was then removed and the cells were washed with HBSS. A 2 ml portion of serum-free DMEM + 0.01% CHAPS or of DMEM containing 5% human serum was added to the flasks containing the confluent cells, and lipopolysaccharide from *Escherichia coli* 055:B5 (10 μg/ml; Sigma, Poole, Dorset, U.K.) was added to half of the flasks in each group. A 1 ml portion of serum-free DMEM + 0.01% CHAPS or of DMEM containing 5% human serum was added to the flasks containing the sub-confluent cells to achieve similar medium/cell ratios to those used for the confluent cells. All flasks were incubated at 37 °C for 24 h. The medium was then removed and divided into batches, and the cells were trypsin-treated and counted as in the cumulative growth-factor-release experiments above except that the medium from the sub-confluent flasks was divided into 0.25 ml portions. Samples were stored at −20 °C before growth-factor assays.

Iodination of bFGF

Recombinant bFGF (rbFGF) (Amersham, Aylesbury, Bucks., U.K.; 5 μg) was iodinated with 1 mCi of $Na^{125}I$ by using chloramine-T, and free ^{125}I separated on a heparin–Sepharose affinity column as previously described [28]. The tracer was repurified on a heparin–Sepharose column immediately before use.

Radioimmunoassay for bFGF

The antiserum used was raised against a synthetic peptide bFGF-(1–24) and was given by Dr. A. Baird (The Whittier Institute, La Jolla, CA, U.S.A.). The antibody was used at a final dilution of 1:20000. rbFGF iodinated as above was used as the label and rbFGF as the standard (9.8–5000 pM). Standards were diluted in serum-free DMEM + 0.01% CHAPS or serum-containing DMEM and all other reagents in 50 mM-phosphate buffer containing 0.15 M-NaCl, 0.025 M-EDTA and 0.2% (w/v) gelatin, pH 7.2. The assay sensitivity was 18 pM, the intra-assay variation 8% and the inter-assay variation 18% at 500 pM. Cross-reactivity in the assay was seen only with acidic FGF (FGF Co. Ltd., La Jolla, CA, U.S.A.) at concentrations greater than 2 nM.

Radioimmunoassay for IGF-1

IGF-1 was measured, after acid/ethanol stripping of samples, as previously described [29]. The polyclonal antiserum UBK 487 was donated by Dr. L. Underwood and Dr. J. J. van Wyck (University of North Carolina, Chapel Hill, NC, U.S.A.) through the National Hormone and Pituitary Program of the National Institute of Diabetes, Digestive and Kidney Diseases. The assay had a sensitivity of 0.3 nM and the inter-assay variation was 8% at 14 nM.

Assay for lactate dehydrogenase (LDH)

LDH was measured kinetically on an RA-XT Analyser (Technicon Instrument Corp., Basingstoke, Hants., U.K.) by a spectrophotometric method optimized to Scandinavian conditions (pyruvate to lactate) at 340 nm [29a].

Bioassay for growth factors

Subconfluent BREC were treated with 0.25% trypsin and a single-cell suspension was prepared. The cells were counted with a Coulter Counter and seeded into each well of 24-multiwell tissue-culture plates, pre-coated with fibronectin, at 2500 cells per well. After the cells had attached overnight in 0.5 ml of DMEM + 10% PDS per well, they were washed once with HBSS and the medium was changed to 0.5 ml of DMEM + 0.1% PDS. After 24 h the cells were again washed with HBSS, and 0.5 ml of DMEM + 0.1% PDS containing 10 μl of [*methyl*-^{3}H]thymidine solution {600 μl of [*methyl*-^{3}H]thymidine (185 GBq/mmol; Amersham), 60 μl of 10 mM-thymidine and 1340 μl of sterile water}/ml was added. Wells were then treated individually by the further addition of either 100 μl of serum-free BREC-conditioned medium harvested from BREC monolayers at days 0–6 as described above, or bFGF (5 pM), and the cells were incubated for a further 48 h. The monolayer was then washed twice with Dulbecco's A phosphate-buffered saline (PBSA) (Oxoid, Basingstoke, Hants., U.K.), and the incorporated [*methyl*-^{3}H]thymidine was precipitated by addition of 5% (w/v) trichloroacetic acid for 5 min, followed by 100% (v/v) ethanol.

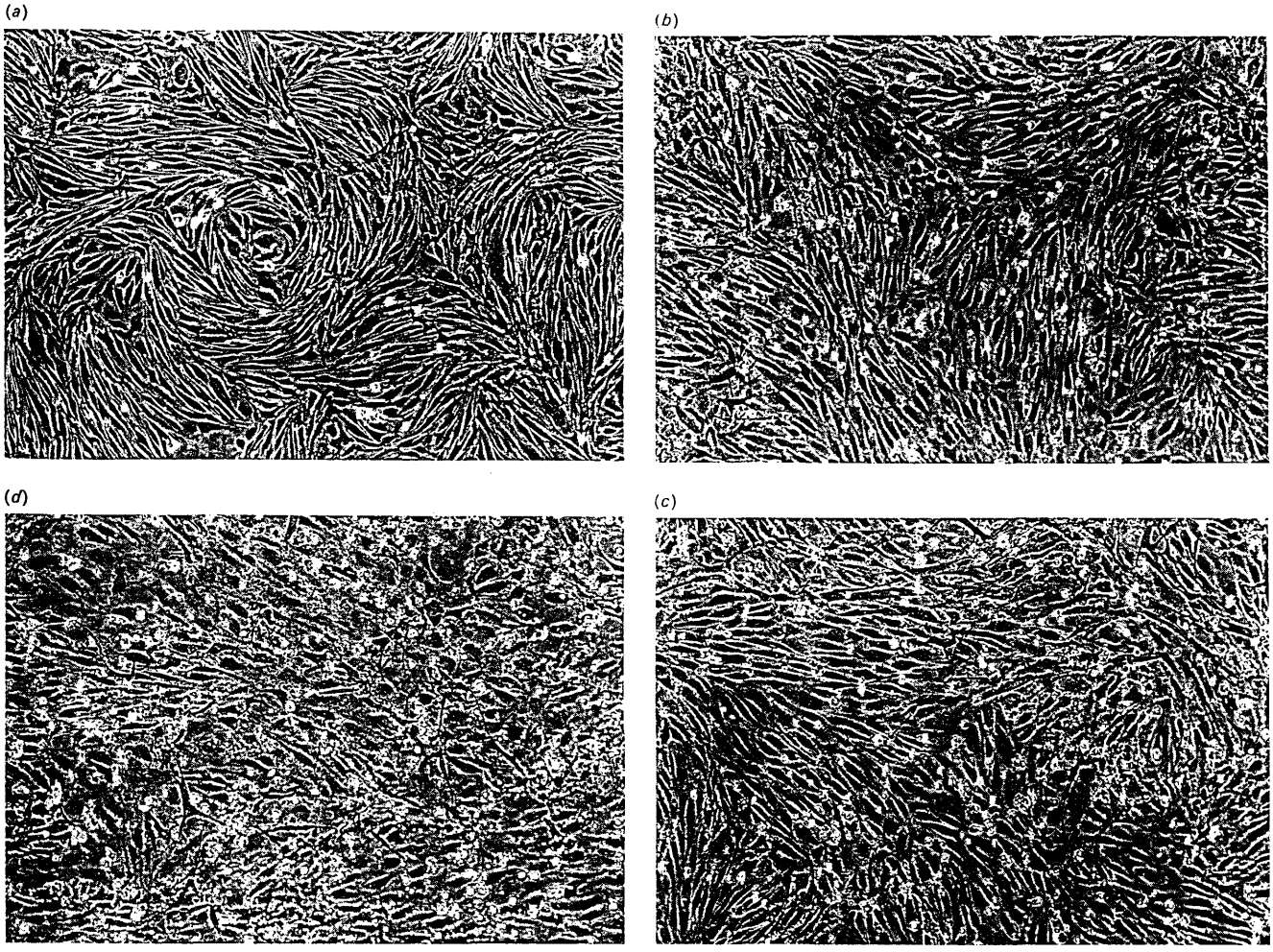


Fig. 1. Changes in the appearance of confluent monolayers of BREC in serum-free DMEM with time

Flasks of confluent BREC used for the serum-free cumulative growth-factor release experiments were photographed daily over the period of the experiment. Results are shown for days 0 (*a*), 2 (*b*), 4 (*c*) and 6 (*d*) after addition of serum-free medium. Magnification × 50.

The ethanol was removed, and the precipitated material was solubilized with 0.5 ml of 25 M-formic acid per well and then placed in 3.5 μl of scintillation fluid (Optiphase Safe) (LKB/Pharmacia, Milton Keynes, U.K.). Each well was rinsed with 0.5 ml of PBSA, which was added to the appropriate scintillation vial. Vials were counted for radioactivity in a Beckman LS 1801 liquid-scintillation counter for 1 min per sample.

Identity of bFGF in BREC-conditioned medium

bFGF in BREC-conditioned medium was characterized by its elution profile on a heparin–Sepharose column compared with that of rbFGF. Heparin–Sepharose was prepared by rehydration and washing in distilled water, followed by pre-equilibration with 0.05 M-Tris/HCl, pH 7.4, containing 0.15 M-NaCl. A column (1.0 cm × 6.0 cm) was packed with heparin–Sepharose, and either 1 ml of rbFGF (305 pM) or 12 ml of BREC-conditioned medium (containing 4.7 pM-bFGF as measured by radioimmunoassay) was run on to the column. Heparin-binding growth factors were eluted from the column with a linear 0.15–3 M gradient of NaCl in 0.05 M-Tris/HCl, pH 7.4, in a total of 120 ml (6 ml/fraction) for rbFGF, or 30 ml (1.5 ml/fraction) for BREC-conditioned medium in a total of 20 fractions. Fractions eluted from the column run with rbFGF were diluted 1:20 with assay buffer for bFGF radioimmunoassay, whereas fractions from the column run with BREC-conditioned medium were dialysed overnight at 4 °C against 0.05 M-Tris/HCl, pH 7.4, and assayed neat against rbFGF standards prepared in 0.05 M-Tris/HCl, pH 7.4, containing 0.15 M-NaCl which had been run through the heparin–Sepharose column before sample application and dialysed.

RESULTS

Effect of serum-free and serum-containing DMEM on BREC monolayers

A confluent monolayer of BREC contained a mean of $6.8(\pm 0.4) \times 10^5$ cells per 25 cm^2 flask ($n = 3$). After 24 h in serum-free medium this number had fallen to $5.2(\pm 0.6) \times 10^5$ cells per flask (Fig. 2). This decrease continued to $4.1(\pm 0.5) \times 10^5$ cells per flask by day 6. The photographic record of the BREC monolayer at zero time and at days 2, 4 and 6 after addition of serum-free DMEM + 0.01 % CHAPS is shown in Figs. 1(*a*)–1(*d*). At zero time the confluent monolayer shows closely packed cells with some cells rounded up on the surface of the monolayer; these appear as bright beads in the phase-contrast micrographs. By day 2 there is an increase in the number of rounded cells in the monolayer, and some of these are seen floating in the

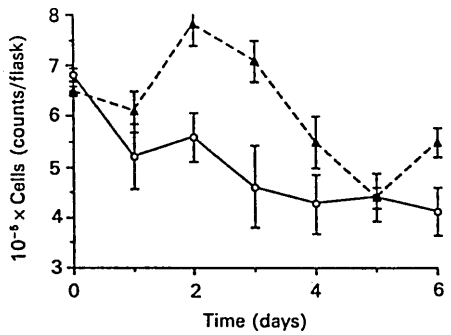


Fig. 2. Change in BREC cell number with time

Cell number per flask in serum-free (○) or serum-containing (▲) culture medium, measured with a Coulter counter (see the text for details), against time. Each point represents the mean ± S.E.M. for three separate experiments.

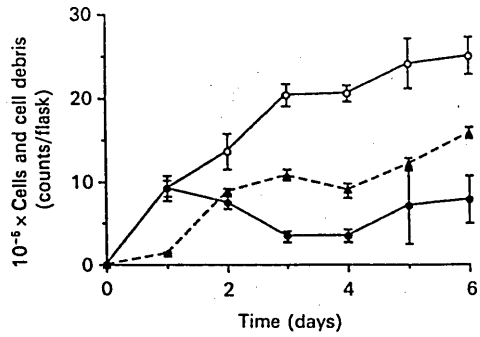


Fig. 3. Cells and cell debris released into the culture medium

Cells and cell debris released as a function of time in serum-free cumulative-release flasks (○), daily-release flasks (●) or serum-containing cumulative-release flasks (▲). Each point represents the mean ± S.E.M. for three separate experiments.

medium. Also several smaller beads and many small dark particles can be seen; these are the components of cell debris. On day 4 the monolayer appears severely disrupted, with many dead cells and cell-debris particles; however, at this stage it is predominantly intact. Finally, on day 6 there is the complete break-up of the monolayer, with large gaps between the endothelial cells, seen on the bottom left of the micrograph (Fig. 1*d*), and an increased amount of cellular debris.

Confluent cell monolayers in medium containing serum showed a different pattern of changes in monolayer cell number compared with that in serum-free medium. There was an initial fall from $6.5(\pm 0.1)\times 10^5$ to $6.1(\pm 0.4)\times 10^5$ cells per flask, but the number then increased to $7.8(\pm 0.4)\times 10^5$ cells per flask after 48 h before falling to the values seen in serum-free medium by day 5. The early increase could be explained by unsustained cell proliferation in response to released growth factors. Monolayer disruption, as viewed through the microscope, was similar to that seen in serum-free medium.

Release of cells from the monolayer and increase in cell debris in serum-free and serum-containing culture medium

There was an increase in the number of cells present in serum-free culture medium as measured with the Coulter Counter (Fig. 3), which coincided with a decrease in cell number in the BREC monolayer. There was a greater rate of increase in particles counted in the medium, $5.2(\pm 0.2)\times 10^5$ counts per flask per day over the first 4 days compared with the decrease in cells in the monolayer of $0.57(\pm 0.15)\times 10^5$ cells per flask per day over the same period. There was also no further decrease in monolayer cell number over the last 2 days, whereas the cell and debris count in the medium continued to increase by $2.3(\pm 0.9)\times 10^5$ counts per flask per day. These results could be partly explained by cell proliferation in the monolayer, but there is also considerable break-up of cells into smaller fragments, as seen in the photographic record (Fig. 1). Cells and cell-debris particles measured in serum-containing culture medium increased at a lower rate [$2.6(\pm 0.6)\times 10^5$ counts per flask] than in serum-free medium, and this could be explained by less cell damage. Monolayer cell number, however, eventually decreased to the levels seen in serum-free medium, indicating that similar monolayer damage did occur in serum-containing medium, and a better explanation is that there was less break-up of released cells into smaller fragments. The number of cells remaining in the BREC monolayer in those flasks where serum-free medium had been removed and replaced daily was $4.0(\pm 0.7)\times 10^5$ cells per flask.

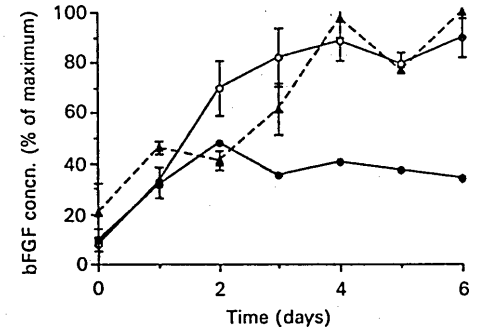


Fig. 4. Release of bFGF into the culture medium

Results are shown as a percentage of the maximum release for each experiment in serum-free cumulative-release flasks (○), daily-release flasks (●) or serum-containing cumulative-release flasks (▲). Each point represents the mean ± S.E.M. for three separate experiments, except for those for the daily-release flasks which show the means of two experiments.

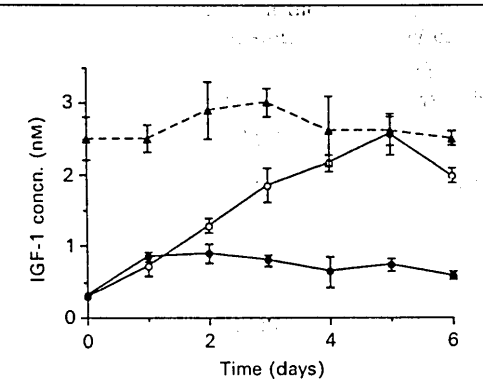


Fig. 5. Release of IGF-1 into the culture medium

Results are shown for serum-free cumulative-release flasks (○), daily-release flasks (●) or serum-containing cumulative-release flasks (▲). Results are expressed as means ± S.E.M. for three separate experiments.

bFGF release into serum-free and serum-containing culture medium

bFGF release from BREC monolayers is shown in Fig. 4. The results are expressed as a percentage of the maximum release of bFGF in each experiment, since the release of bFGF from BREC monolayers varied depending on the batch of cells used. The rate

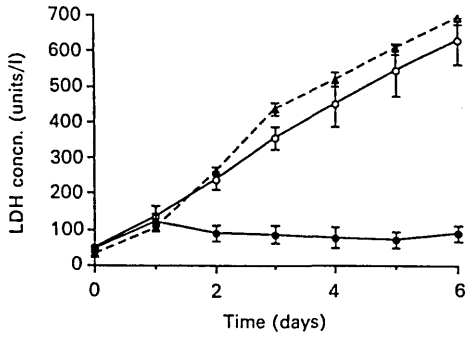


Fig. 6. Release of LDH into the culture medium

LDH release into the medium by confluent cultures of BREC as a function of time is shown for serum-free cumulative-release flasks (○), daily-release flasks (●) or serum-containing cumulative-release flasks (▲). Results are expressed as means ± S.E.M. for three separate experiments.

of release of bFGF over the first 4 days in each of three experiments was 31.8, 71.0 and 105.2 pM/day in the serum-free cumulative-release flasks, reaching a maximum of 149, 476 and 467 pM respectively. bFGF was released into serum-containing culture medium at rates of 49.0, 61.3 and 54.3 pM/day over the first 4 days, and reached a maximum of 224, 280 and 315 pM respectively. bFGF was released at rates of 49 and 177 pM/day in the daily-release flasks.

IGF-1 release into serum-free and serum-containing culture medium

The results for IGF-1 release are shown in Fig. 5; all results are means ± S.E.M. of data from three separate experiments. IGF-1 was released into serum-free DMEM by BREC monolayers at a rate of 0.34 ± 0.02 nM/day, reaching a maximum of 2.56 nM. The release rate of IGF-1 in the daily-release flasks was 0.75 ± 0.05 nM/day. A baseline level of IGF-1 of 2.50 ± 0.28 nM, owing to the presence of IGF-1 in serum, was seen in serum-containing culture medium, and there was no significant increase in IGF-1 concentration over the time course of the experiment.

LDH release into serum-free and serum-containing culture medium

The results for LDH release are shown in Fig. 6; all results are means ± S.E.M. of data from three separate experiments. LDH release into serum-free DMEM by BREC followed a rate of 105 units/litre per day, to reach a maximum of 628 units/litre. A constant release of 90 ± 7 units/litre per day was seen in the same monolayer when fresh medium was added daily. LDH was released into serum-containing medium at a rate of 110 units/litre per day, reaching a maximum of 689 units/litre. This was similar to the pattern of release seen in serum-free medium, indicating a comparable amount of cell lysis in cell monolayers maintained with or without the addition of serum.

Growth-factor release from lipopolysaccharide-treated and sub-confluent BREC monolayers

bFGF release after 24 h from sub-confluent BREC and confluent cells with or without lipopolysaccharide (10 μg/ml) into both serum-free and serum-containing medium is shown in Fig. 7. Also shown are the corresponding changes in IGF-1 concentration, LDH concentration, cell and cell-debris particles in the medium and monolayer cell number. All columns represent means ± S.E.M. of data from at least three experiments, except where indicated. In confluent flasks at 24 h, bFGF concentrations were 91 ± 24 pM in serum-free medium and 161 ± 35 pM in

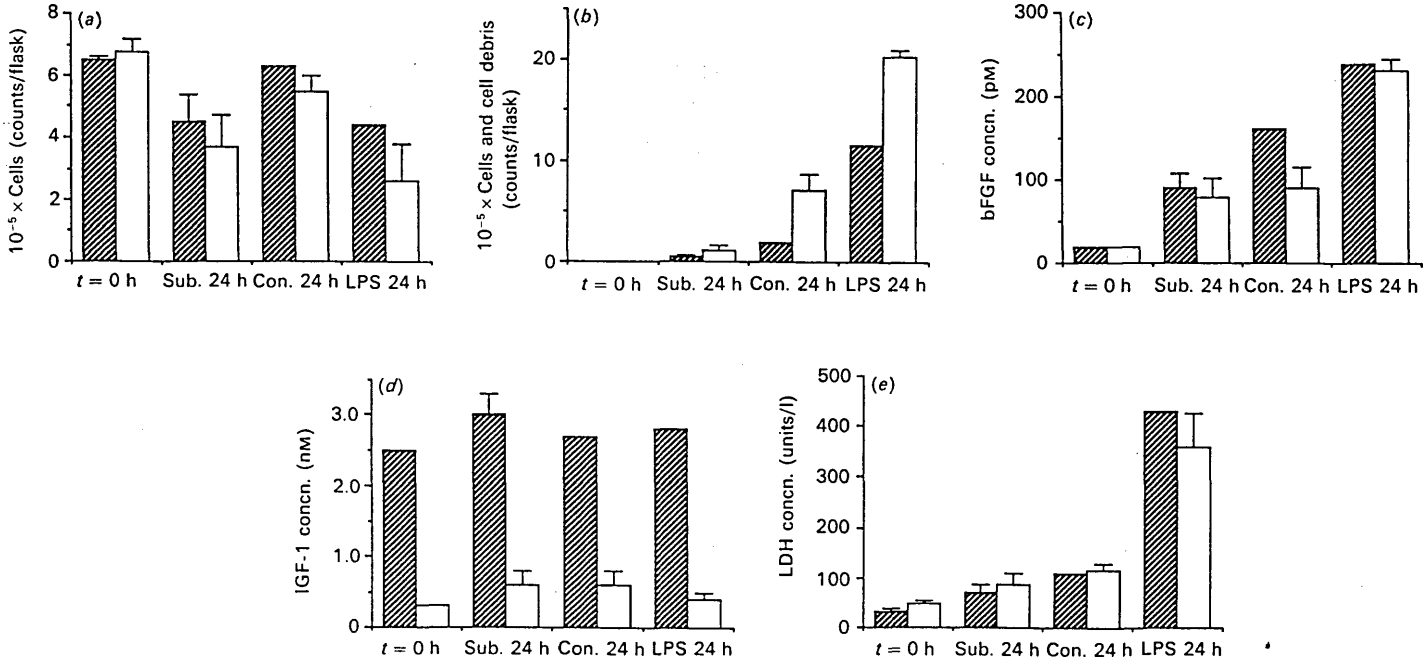


Fig. 7. Growth-factor release and changes in parameters of cell damage in sub-confluent BREC monolayers and confluent monolayers treated with lipopolysaccharide

Results are shown as the means for (*a*) monolayer cell number, (*b*) cell and cell-debris-particle numbers, (*c*) bFGF concentration, (*d*) IGF-1 concentration and (*e*) LDH concentration in serum-free medium (□) or serum-containing medium (▨) at zero time (t = 0) and at 24 h in flasks containing sub-confluent cells (Sub. 24 h), confluent cells (Con. 24 h) or confluent cells treated with lipopolysaccharide (10 μg/ml) (LPS 24 h). Error bars represent the S.E.M. for at least three separate experiments, except for results from serum-containing confluent cells at 24 h and treated with lipopolysaccharide where two and one experiments were carried out respectively.

Table 1. Bioactivity of BREC-conditioned medium

[*methyl*-^{3}H]Thymidine uptake into BREC was measured in 24-well tissue-culture plates (see the text for details) in the presence of serum-free BREC-conditioned medium removed from BREC monolayers at zero time and every day for 6 days. Uptake in response to rbFGF (5 pM) is shown as a control. Results are means ± S.E.M. of data from six separate experiments. Values were compared by one-way ANOVA ($P < 0.01$) and then each value was compared with that at zero time by an unpaired t test ($P < 0.05$ in every case).

Addition to cell-culture well	[*methyl*-^{3}H]Thymidine uptake (d.p.m.)
100 μl of BREC-conditioned medium harvested at:	
0 days	7550 ± 479
1 day	10467 ± 924
2 days	11307 ± 783
3 days	10210 ± 543
4 days	11171 ± 985
5 days	12075 ± 760
6 days	10073 ± 417
rbFGF (5 pM)	10678 ± 343

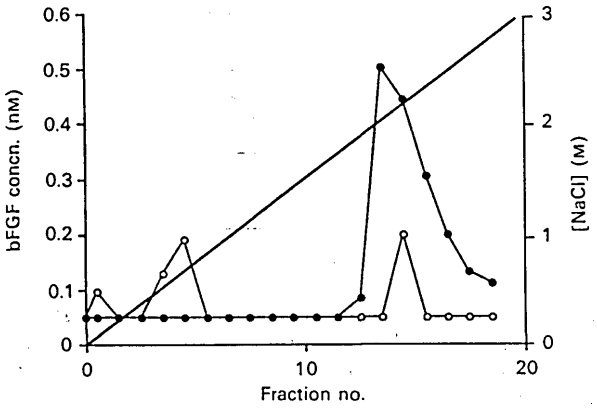


Fig. 8. Heparin–Sepharose profile of rbFGF and BREC-conditioned medium

rbFGF (●) and BREC-conditioned medium (○) were eluted from a heparin–Sepharose column (see the Materials and methods section for details) with a gradient of increasing NaCl concentration (——; 0–3 M). bFGF in the fractions was measured by radioimmunoassay.

medium containing serum. These were increased to 230 ± 14 and 238 pM respectively when the cells were treated with lipopolysaccharide (10 μg/ml). The decrease in monolayer cell number and increase in cell and cell-debris particles measured in the medium were also greatest when the cells were treated with lipopolysaccharide. The number of cell and cell-debris particles measured in serum-containing medium after 24 h was less than that seen in serum-free medium. This was also observed in the cumulative-release experiments, and was probably due to less cell break-up in the presence of serum. LDH release, however, was similar into both serum-free (116 ± 13 units/litre) and serum-containing (109 ± 5 units/litre) medium, and was substantially increased after addition of lipopolysaccharide, to 360 ± 56 and 428 units/litre respectively. These results suggest that, although the amount of cell debris was lower in serum-containing medium, cell lysis, as measured by LDH release, was just as great. IGF-1 concentrations did not show any change in serum-containing medium from the baseline level of 2.50 ± 0.28 nM and, although there was a small increase in serum-free medium, from 0.32 to 0.6 ± 0.3 nM, there was no increase when the cells were treated with lipopolysaccharide.

bFGF was released into serum-free and serum-containing culture medium by sub-confluent BREC, reaching levels of 85 ± 23 and 70 ± 16 pM respectively. Cell number at 24 h was similar, 3.7 (± 1.0) × 10^5 and 4.5 (± 0.9) × 10^5 cells per flask, and, although there were few cell and cell-debris particles in the medium [1.2 (± 0.7) × 10^5 and 0.5 (± 0.1) × 10^5 counts per flask], LDH release (85 ± 23 and 70 ± 16 units/litre) was almost as much as from confluent cells. This indicates that cell lysis had occurred, and this could account for the release of bFGF. There was no significant release of IGF-1 from sub-confluent cell monolayers.

Bioactivity of BREC-conditioned medium

Medium from serum-free cumulative-release flasks sampled on days 1–6 produced increased [^{3}H]thymidine uptake into BREC as compared with medium sampled at zero time (Table 1). bFGF (5 pM) also gave a significant stimulation of [^{3}H]thymidine uptake.

Identity of bFGF in BREC-conditioned medium

To confirm the identity of the bFGF measured in the radioimmunoassay, the heparin–Sepharose elution profile of BREC-conditioned medium was compared with that of rbFGF. The rbFGF was eluted at 1.9 M-NaCl, reaching a peak at 2.2 M. Recovery as a percentage of rbFGF applied to the column was 34.3 %. BREC-conditioned medium from serum-free cumulative-release flasks was eluted as three peaks at 0.15 M-, 0.8 M- and 2.3 M-NaCl, the final peak corresponding to the elution position of rbFGF (Fig. 8).

DISCUSSION

Retinal endothelial-cell proliferation is a requirement for new vessel growth as seen in proliferative diabetic retinopathy. The stimulus to this proliferation has not been determined, although the development of large areas of ischaemic retina usually precedes this event. We have shown that in monolayer culture BREC release large quantities of bFGF, which is a potent mitogen for these cells. The maximum release of bFGF from these monolayers into serum-free medium is 105.2 pM/day and into serum-containing medium is 61.3 pM/day; this compares with an ED_{50} for the stimulation of BREC proliferation by rbFGF of 2.8 pM (R. A. Brooks, unpublished work) and by purified bFGF of 0.8 pM [11] and 5.5 pM [24]. The amount of bFGF released by BREC monolayers is therefore in excess of that required to bring about retinal endothelial-cell proliferation. We have also shown that serum-free DMEM + 0.01 % CHAPS conditioned by BREC monolayers is mitogenic for BREC, and this ability to stimulate BREC proliferation is similar to that shown by rbFGF (5 pM).

The lack of a classic signal-peptide sequence on the mRNA transcript coding for bFGF, which would allow secretion, has led to speculation as to the source of bFGF measured in endothelial-cell-conditioned culture medium. Several authors have suggested that bFGF is mainly stored in the extracellular matrix, where it is bound to heparan sulphate proteoglycans [15,16,18,21] and can be released by heparinitase and heparin [15,22]. Others have shown release of bFGF in viable confluent cultures of bovine corneal endothelial cells [30] and BREC [24] by an unknown mechanism. Rifkin & Moscatelli [31] have pointed out that, even in viable cultures, there is a small amount of cell death, and this could be responsible for the measurable bFGF. Endothelial cells from bovine aorta, adrenal capillaries

and retinal vessels have been shown to contain extractable bFGF [17,24]. Using bovine aortic endothelial cells, Gajdusek & Carbon [23] demonstrated the presence of large quantities of bFGF, which can be released by cell lysis and then bind to extracellular matrix, where it could be released by heparin and the enzyme heparinitase.

We have now shown the release of bFGF from bovine retinal endothelial cells into both serum-free and serum-containing medium and related this to parameters of cell damage and lysis. In serum-free medium, bFGF increases along with a decrease in cell number in the monolayer. Its release is also associated with increased cells and cell debris in the culture medium and with release of the enzyme LDH, which provides a marker of cell lysis [32]. The quantity of bFGF released was variable and was dependent on the batch of cells used. The quantity of cells and debris in the medium increased more rapidly than the decrease in monolayer cell number. This could be due to either cell proliferation in the monolayer or degradation of released cells, and, although the former cannot be ruled out, the appearance of small particles in the conditioned medium indicates that cell break-up did occur. Additionally, the serum-free medium in these flasks would not readily support the proliferation of retinal endothelial cells even in the presence of released growth factors. In serum-containing medium, bFGF release can also be linked to parameters of cell damage and lysis. Although there is an initial increase in monolayer cell number and a less marked linear increase in cell debris in the medium, owing to less cell break-up, LDH release was similar to that seen in serum-free medium, indicating that cell lysis did occur. The fact that LDH concentration increases linearly to day 6 in both serum-free and serum-containing medium, whereas bFGF release forms a plateau after day 4, is probably due to progressive degradation of bFGF by proteases released from damaged cells, leaving it unable to bind to the antibody in the radioimmunoassay.

In order to investigate more rigorously the relationship between cell damage, lysis and bFGF release, cell growth conditions were chosen to produce minimum (sub-confluent cells) and maximum (lipopolysaccharide-treated cells) cell damage. Endotoxin-mediated cell damage has been used previously to investigate the release of growth-promoting activity from bovine aortic endothelial cells [23]. Release of bFGF in both serum-free and serum-containing medium was greatest when cells were treated with lipopolysaccharide, which also produced the largest decrease in monolayer cell number and increase in cell debris and LDH concentration in the medium. A lower but significant release of bFGF was also seen when serum-free or serum-containing DMEM was conditioned by sub-confluent BREC monolayers. Although there were few cell-debris particles released into the medium, indicating little cell break-up, LDH was released in quantities approaching those seen from confluent monolayers, showing that cell lysis had occurred. This would provide an explanation for bFGF release. Insufficient extracellular matrix was produced by these cells, under the experimental conditions used, to allow extraction and measurement of bFGF. The absence of this means of bFGF sequestration may have increased the measurable concentration of this growth factor in BREC-conditioned medium.

The identity of bFGF released from the monolayer was shown by heparin–Sepharose affinity chromatography. The concentration of NaCl at which both rbFGF and bFGF in BREC-conditioned medium are eluted from the column is higher than that observed by other investigators [17,33]. However, these values have been shown to be very dependent on the batch of heparin–Sepharose used [34]. Two other peaks of bFGF immunoreactivity were observed in the elution profile of BREC-conditioned medium, at 0.15 M- and 0.8 M-NaCl. The first of these could correspond to the weakly bound high-molecular-mass fraction observed by Courty *et al.* [6] in retinal extract and the second to acidic FGF. If this were due to cross-reactivity of acidic FGF in the radioimmunoassay, then it would imply very high concentrations of acidic FGF in BREC-conditioned medium. Baird *et al.* [35] have reported that bFGF can be co-eluted with a low-salt fraction from a heparin–Sepharose column, and this is a more likely explanation.

At the end of each experiment, the flasks to which serum-free DMEM + 0.01 % CHAPS had been added daily showed more disruption to the BREC monolayer than in the flasks used for measuring serum-free cumulative growth-factor release, but the number of cells remaining in the monolayer was similar. The cumulative cell and debris count in the serum-free medium and the LDH levels were the same in both sets of flasks, as was IGF-1 release until levels reached a plateau. This plateau could be due to a cessation of IGF-1 release in these flasks or to degradation of IGF-1 by proteases released from the lysed cells.

IGF-1 may contribute to the retinal endothelial-cell proliferation seen in proliferative diabetic retinopathy. It has been shown by King *et al.* [36] to be mitogenic for retinal endothelial cells in culture, and it can interact with bFGF in stimulating the progression of cells through the cell cycle [37]. Hyer *et al.* [38] showed a transient rise in the serum level of IGF-1 in diabetics at the time of new vessel growth, which returned to normal levels after successful laser treatment, suggesting a role for IGF-1 *in vivo*. We have shown that IGF-1 is released from BREC monolayers into serum-free medium; however, there was no release into serum-containing medium or when the cells were damaged by the addition of lipopolysaccharide. These results suggest that IGF-1 is not released from damaged BREC. The fact that IGF-1 can be conventionally secreted from cells [39] suggest this as a likely explanation for its release into serum-free medium. In serum-containing medium, there may be negative feedback on release owing to the presence of IGF-1 in the serum.

Both bFGF and IGF-1 have been reported to be elevated in the vitreous of patients with proliferative diabetic retinopathy [40,41]. Our results show that both of these growth factors can be released from BREC, but that their mechanism of release is different. IGF-1 is likely to be secreted, whereas bFGF is released after cell damage and lysis, although this does not preclude additional release of bFGF from viable cells by an as yet unknown mechanism. Retinal capillary damage is a feature of advanced diabetic retinopathy, and further endothelial-cell damage could occur in the ischaemic retina before neovascularization. Although not wishing to exclude the contribution of growth factors released from other retinal cells, our results are further evidence for the involvement of bFGF in this sight-threatening condition.

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Rapid Publication

Production of Endothelin 1 by Cultured Bovine Retinal Endothelial Cells and Presence of Endothelin Receptors on Associated Pericytes

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Endothelinlike immunoreactivity was detected by radioimmunoassay in medium conditioned by cultured endothelial cells obtained from bovine retinal microvessels (9.2 ± 6.5 pM, n = 4). Sephadex G-25 column chromatography and fast-protein liquid chromatography revealed that most of the endothelinlike immunoreactivity was eluted in an identical position to synthetic endothelin 1. Retinal capillary pericyte–conditioned medium contained 2.9 pM endothelinlike immunoreactivity. In contrast to endothelial cells, retinal pericytes were found to bind endothelin. The dissociation constant and binding capacity were 0.14 nM and 1.5 × 10^5 sites/cell (n = 3), respectively. These findings suggest that endothelin produced by the retinal endothelial cells binds to the pericytes, adding support to the suggestion that pericytes in the retina may have a musclelike function. ***Diabetes*** **38:1200–202, 1989**

Endothelin 1 is a potent vasoconstrictor peptide that was originally isolated and structurally characterized from the conditioned medium of porcine aortic endothelial cells (1). Endothelin binding sites have been shown in cultured rat vascular smooth muscle cells (2). Three endothelin genes (for endothelin 1, 2, and 3) exist in the human, porcine, and rat genome (3). Endothelin is thought to be produced by endothelial cells of the major arteries, but it has not been established whether endothelin is produced in microvascular circulation.

To help clarify the physiological role of endothelin in retinal microcirculation and its involvement in the genesis of diabetic retinopathy, we investigated the production of endothelin and the presence of endothelin binding sites on cultured bovine retinal endothelial cells and pericytes.

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RESEARCH DESIGN AND METHODS

Cell culture. Bovine retinal endothelial cells (BREC) were isolated and cultured as previously described (4). Briefly, retinas were dissected from freshly slaughtered cattle, homogenized in minimum essential medium, and filtered through an 85-μm filter. The trapped microvessels were digested in a 1-mg/ml collagenase-dispase (Boehringer Mannheim, Mannheim, FRG) solution for 65 min at 37°C and plated onto a fibronectin substrate in 75-cm^3 tissue-culture flasks. The culture medium was 8% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Tryptose phosphate broth (Gibco, Paisley, UK) and 10% plasma-derived serum. All culture mediums were purchased from Gibco and tissue-culture plastics from Falcon (Becton Dickinson, Oxford, UK). Bovine retinal pericytes (BRP) were cultured from microvessels prepared like the endothelial cells, but the vessels were plated at a lower density onto the plastic substrate of a 75-cm^3 tissue-culture flask. Supplemented DMEM was also used for BRP, but 20% fetal calf serum replaced the plasma-derived serum. Both cell types were grown to confluence in sealed flasks gassed with 95% air/5% CO_2.

Both cell types were characterized by their morphology. BREC displayed contact inhibition and had a typical cobblestone appearance. BRP had many cytoplasmic processes and overgrew at confluence. BREC showed uniform staining for factor VIII–related antigen, whereas BRP did not stain.

At confluence, the cells were trypsinized. After washing twice with Hank's buffered salt solution (HBSS), 0.25% trypsin was added. When the cells had detached from the substrate (1.5 min for BREC and 10 min for BRP), the trypsin was neutralized by the addition of serum-containing medium. For preparation of conditioned medium, trypsinized cells were plated into 75-cm^3 tissue-culture flasks and grown to confluence as described. At confluence, medium was removed, the cells were washed with HBSS, and 5 ml of either serum-containing DMEM or serum-free DMEM was added. After 2 days, this medium was harvested and used for measurement and characterization of endothelin.

For binding studies, trypsinized cells were seeded into 24-well tissue-culture plates that had been coated with fibronectin for BREC but not for BRP. They were grown to confluence in 0.8 ml serum-containing DMEM/well before use.

Endothelin characterization. Endothelinlike immunoreactivity (ELI) in conditioned medium was extracted by Sep-Pak C18 cartridges (Waters, Milford, MA). Eight milliliters of conditioned medium acidified with 4 ml of 4% acetic acid was loaded onto a cartridge, and ELI was eluted with 2 ml of 60% (vol/vol) acetonitrile/water containing 0.026 M ammonium acetate. The eluate was dried in a Savant vacuum centrifuge, the resulting pellet reconstituted in assay buffer (60 mM PO_4 buffer, pH 7.4, containing 10 mM EDTA, 7 mM sodium azide, and 0.3% (wt/vol) bovine serum albumin [BSA]), and the aliquot assayed in duplicate. The mean ± SD recovery of this extraction procedure was 95 ± 8.9% (n = 6).

The antiserum to endothelin 1 was raised in a rabbit with synthetic endothelin 1 (Nova Biochem, Nottingham, UK) conjugated with BSA by the diazo technique (5). The antiserum produced was used in the assay at a final dilution of 1:35,000. Synthetic endothelin 1 (Peptide Institute, Osaka, Japan) was used as standard and for iodination with ^{125}I-labeled sodium by the chloramine-T method (6) and purified by high-performance liquid chromatography as previously described (7). After 3 days of incubation at 4°C, antibody-bound and free fractions were separated by charcoal adsorption of the free fraction.

The sensitivity of this assay was 0.5 fmol/tube at 95% confidence. The assay showed 23% cross-reaction with endothelin 2 and 20% with endothelin 3 (Peptide Institute) but no cross-reaction with other known peptides. Intra- and interassay coefficients of variation were 12% (n = 9) and 19% (n = 7), respectively.

Fractionation of ELI in the medium extract was carried out by Sephadex G-25 column chromatography (0.9 × 58 cm, superfine) and fast-protein liquid chromatography (FPLC) with a high-resolution reverse-phase (Pep RPC HR 5/5) C-18 column (Pharmacia, Uppsala, Sweden) with a gradient of acetonitrile from 15 to 35% (vol/vol) in water over 1 h at 1 ml · min^{-1} · fraction^{-1}.

Binding experiments. Confluent 24-well plates (7 × 10^4 BREC/well or 4 × 10^4 BRP/well) were used for binding assays. Cells were incubated at 37°C for 90 min with 0.05 nM ^{125}I-labeled endothelin 1 in Earl's balanced salt solution containing 10 mM HEPES and 0.3% BSA. After incubation, cells were extensively washed and solubilized in 1 M NaOH. The cell-bound radioactivity was determined in a γ-counter. Specific binding was obtained by subtracting from total binding the nonspecific binding in the presence of an excess (0.1 μM) of unlabeled endothelin 1. Scatchard plots were obtained by adding increasing amounts of labeled endothelin, and the results were analyzed by linear regression. IC_{50} values were obtained by adding increasing concentrations of endothelin 1, 2, and 3.

RESULTS

ELI was detected in the BREC-conditioned culture medium (mean ± SE 9.2 ± 6.5 pM, n = 4), whereas no ELI was detected in the unconditioned medium (<0.3 pM). The ELI in the BRP-conditioned culture medium was 2.9 pM.

Column chromatography and FPLC showed that most ELI eluted in an identical position to synthetic endothelin 1, whereas there was none in the position of endothelin 2 and little in the position of endothelin 3 (Fig. 1). The recovery of ELI in the conditioned medium from column and liquid chromatography was 107 and 73%, respectively.

Endothelin binding to BREC and BRP reached equilibrium between 90 and 120 min (data not shown). The binding sites in BRP were saturable (Fig. 2*A*). The number of binding sites in BRP was 1.5 × 10^5 sites/cell, and the K_d was 0.14 nM (n = 3). Compared with BRP, binding in BREC was very low (<2000 sites/cell), and reliable measurements of B_{max} and K_d could not be made (data not shown). Binding was specific in BRP and was not displaced by unrelated peptides. Endothelin 2 and 3 showed a lower affinity for the receptors compared with endothelin 1. The IC_{50} of endothelin 1 and 2 was 0.56 and 1.53 nM (n = 3), respectively. Endothelin 3 caused only 55% displacement of label at concentration of 1 μM (Fig. 2*B*).

DISCUSSION

This study showed that endothelin 1 is present in the conditioned medium of cultured BREC, and endothelin binding

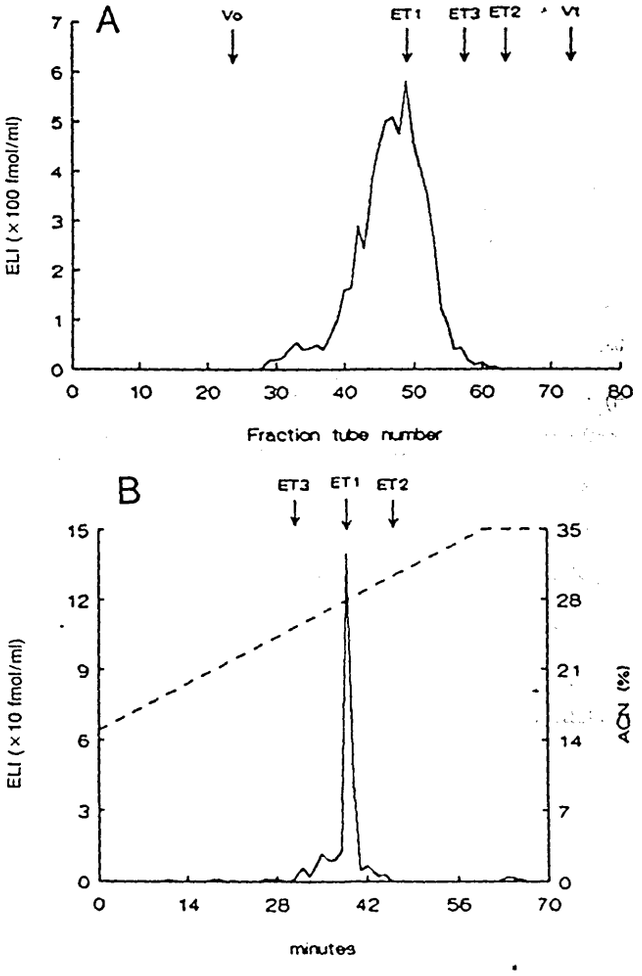


FIG. 1. Sephadex G-25 column chromatography (*A*) and fast-protein liquid chromatography (*B*) of conditioned medium of cultured bovine retinal endothelial cells. ELI, endothelinlike immunoreactivity; Vo, void volume; Vt, total volume; ET1, ET2, and ET3, elution positions of endothelin 1, 2, and 3, respectively; ACN, acetonitrile.

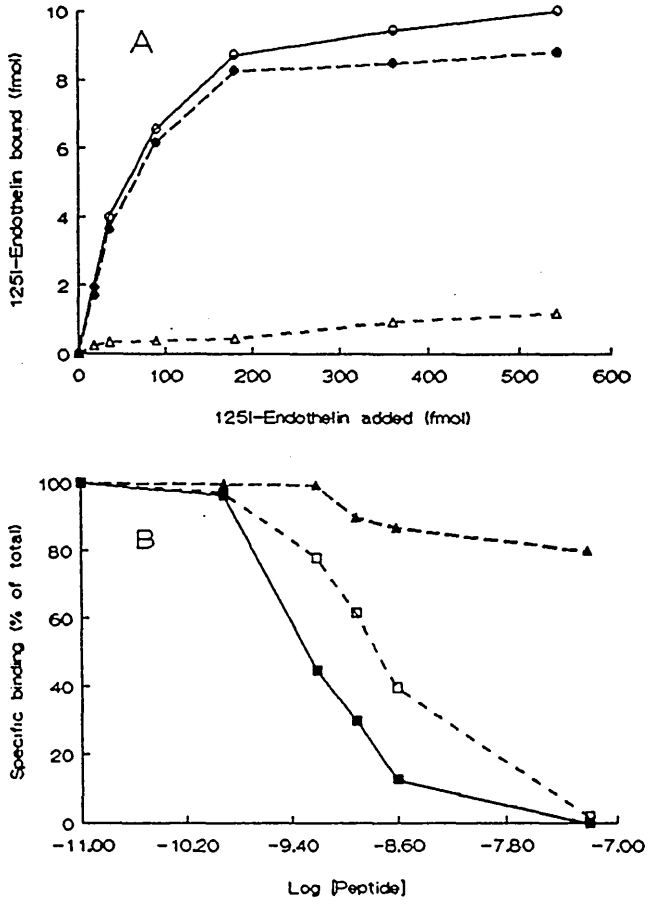


FIG. 2. *A*: saturation of endothelin binding sites in bovine retinal pericytes by adding increasing concentration of ^{125}I-labeled endothelin. ○, Total binding; ●, specific binding; △, nonspecific binding. *B*: displacement of ^{125}I-labeled endothelin binding sites in bovine retinal pericytes by increasing concentration of endothelin 1 (■), endothelin 2 (□), and endothelin 3 (▲).

sites are present in cultured BRP. FPLC revealed that most of the ELI in the BREC-conditioned culture medium eluted in an identical position to synthetic endothelin 1, and little eluted in the position of endothelin 3. The antiserum to endothelin 1 used in this study showed 20% cross-reaction with endothelin 3, therefore it is possible that a greater amount of endothelin 3 may be present in the conditioned medium than was shown by FPLC analysis.

The finding of endothelin was initially unexpected, because embryologically the retina is an outgrowth of the brain, and preproendothelin mRNA has not yet been reported in brain microvessels (1). If endothelin is present in human retinal microvessel endothelial cells, it may be important in the evolution of some retinal vascular diseases, especially diabetic retinopathy.

The exact role of pericytes in the retina has not been clearly established, but a recent study by Orlidge and D'Amore (8) suggested that pericytes have a controlling influence on endothelial cell growth and multiplication. A muscle cell–like action for pericytes was put forward by Kuwabara and Cogan (9) in the 1960s who suggested that capillary dilation with endothelial proliferation and shunt-vessel formation seen in diabetic retinopathy after pericyte loss was due to the loss of this action (10). This possibility was strengthened by Hohman et al. (11), who showed the presence of muscle actin in human retinal pericytes, and Henkind (12), who suggested that pericytes may be capable of differentiating into smooth muscle cells under certain conditions.

In most tissues, endothelin acts on the smooth muscle cells of the vascular wall. The fact that in the retina it may act on pericytes would further emphasize the musclelike function of these cells and explain the dilation of capillaries seen in early diabetic retinopathy by the time pericyte loss occurs (13). Loss of endothelin action could therefore play an important part in the development of diabetic retinopathy.

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Serum IGF-1 Concentration in Diabetic Retinopathy

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Growth factors such as growth hormone and insulin-like growth factor 1 (IGF-1) may be important in the pathogenesis of diabetic retinopathy. We measured serum IGF-1 in 371 diabetic patients attending a diabetic retinopathy clinic and in 73 non-diabetic control subjects. No significant difference was observed in IGF-1 level between the diabetic and control groups (168 ± 3.9 vs 177 ± 7.4 μg/l [mean ± SE]). Within the diabetic group, there was no difference between patients with no retinopathy and those with proliferative change (198.7 ± 8.8 vs 190.5 ± 11 μg/l). After adjusting for differences in age, duration of diabetes, and presence of proteinuria, only the inactive previously proliferative group showed any significant difference from the other patient subgroups (151.8 ± 11.5 μg/l; $p<0.05$). Serum IGF-1 correlated with age in the control group ($r = -0.49$; $p<0.001$) and to a lesser extent in the diabetic group ($r = -0.23$; $p<0.05$). IGF-1 levels were higher in patients with proteinuria than in those without proteinuria (196.8 ± 10.3 vs 138.8 ± 4.4 μg/l; $p<0.001$).

KEY WORDS Insulin-like growth factor 1 Diabetic retinopathy Growth hormone Radioimmunoassay

Introduction

Insulin-like growth factor 1 (IGF-1), now known to be identical with somatomedin C, is the major circulating growth hormone (hGH) dependent growth factor in man and mediates the growth-promoting actions of hGH.[1,2] Since hGH has been postulated to be an important factor in the development of proliferative retinopathy in diabetes,[3-8] it is of interest to investigate whether IGF-1 concentrations correlate with severity of retinopathy, both for the understanding of the pathogenesis of this serious diabetic complication and for possible therapeutic interventions.

Published studies on IGF-1 levels in diabetes have shown normal,[9,10] high[11] or low[12,13] values, reflecting differences in number and selection of patients and difference in methods of estimating IGF activity (bioassay, radioimmunoassay, radioreceptor assay). The picture is even less clear with regard to levels in patients with diabetic retinopathy, levels having been reported as high[14,15] or normal.[9,16]

In this study, IGF-1 values were compared in normal subjects and in patients with varying degrees of diabetic retinopathy. We also examined the relationship of IGF-1 to various relevant clinical parameters.

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Patients

Seventy-three non-diabetic normal subjects chosen fror amongst staff members and 371 diabetic patients attendin the Diabetic Retinopathy clinic at Hammersmith Hospit were studied. Patients with serum creatinine greater tha 120 μmol/l were excluded from the study to eliminat overt renal failure as a variable. Clinical characteristics the subjects are shown in Table 1. Diabetic patients we divided into the following subgroups: 1. no retinopath (84 patients); 2. background changes (92 patients 3. preproliferative changes defined as the appearance multiple haemorrhages associated with venous change cotton wool spots and intraretinal microvascular abno malities (58 patients); 4. active proliferative retinopath defined by the appearance of retinal new vessels with fibrosis (65 patients); and 5. previously active but no quiescent proliferative retinopathy defined as the appea ance of inactive retinitis proliferans or an effective treated retina without new vessels (72 patients). Patie in subgroup (4) were currently receiving laser thera while those in subgroup (5) were already effectiv treated. Retinopathy was assessed by an ophthalmolog on the basis of direct and indirect ophthalmosco colour photography, and fluorescein angiograp Assessment was made without knowledge of IGF levels

Patient and control subjects were similar in respect male:female ratio and body mass index. The non-diab

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Table 1. Characteristics of patients and control subjects studied

Group	Male/ female	Age (years)	BMI (kg/m^2)	Insulin/ no insulin	Duration of diabetes (years)	HbA_1 (%)	Proteinuria (yes/no)	Random blood glucose (mmol/l)
Control	41/32	37±1	22.9±2.4	-	-	5.0–8.0	-	3.0–5.0
All diabetic	195/138	49±1	27.5±0.9	219/127	16.1±0.5	10.3±0.1	57/175	12.5±0.2
No retinopathy	50/34	39±2[a]	25.6±0.5	44/17	7.8±0.7[b]	10.3±0.1	0/46*	12.9±0.4
Background retinopathy	42/35	50±2	30.0±2.6	66/25	19.8±0.9	10.1±0.1	15/47	11.7±0.4
Pre-proliferative retinopathy	38/18	54+2	27.3±0.9	25/33	14.8±1.1	10.8±0.3	17/20*	13.4±0.8
Active proliferative retinopathy	25/18	48±2	27.3±0.5	38/25	17.0±0.9	9.7±0.2[a]	11/19[a]	13.1±0.6
Previous proliferative retinopathy	40/33	54±2	27.0±0.5	46/27	21.3±1.2[a]	10.4±0.2	14/43	11.5±0.6

Mean ± SE, [a]$p<0.05$, [b]$p<0.001$, compared with other diabetic subgroups.

conrol subjects were significantly younger than the diabetic subjects ($p<0.05$). Within the diabetic group, those with no retinopathy were significantly younger than the other subgroups ($p<0.001$). They also had a shorter duration of diabetes than the other patients ($p<0.001$).

Methods

Non-fasting blood samples were taken from subjects between 0900 and 1200 h during clinic time for measurement of IGF-1, blood glucose and glycosylated haemoglobin (HbA_1). A single urine sample was obtained in 232 patients for detection of protein by Albustix.

Four patients with active proliferative retinopathy and four normal subjects were admitted to the Metabolic Unit for 24-h profiles of growth hormone (hGH) and IGF-1. The purpose of this was to assess the variability of levels over 24 h and the validity of measuring single samples as representative of 24-h IGF-1 levels particularly in subjects with hGH hypersecretion.

Serum hGH was measured by double antibody radioimmunoassay (RIA) using antiserum RD16 (Wellcome Diagnostics, Beckenham, UK). Serum IGF-1 was also measured by double antibody RIA using the monoclonal antiserum 3D 1/2/1 supplied by Dr R. C. Baxter through the National Hormone and Pituitary Program.[17] The standard was synthetic IGF-1 (Amersham International, Amersham, UK) and this was labelled with ^{125}I using chloramine-T.

Before measurement, samples were acid ethanol stripped to separate IGF-1 from its binding protein.[18] At a concentration of 200 μg/l, the intra- and inter-assay coefficients of variation for IGF-1 were 5.3 and 10.2 %, respectively. Plasma glucose was measured by the glucose oxidase method using the Beckman Glucose Analyser (Beckman, Fullerton, California, USA). Glycosylated haemoglobin was measured by cellulose acetate membrane electrophoresis (Corning, Palo Alto, California, USA); normal range 5.0–8.0 %.

Analysis

Statistical comparisons between multiple groups were made by analysis of variance (BMDP IV Software, Los Angeles, California, USA) and analysis between two groups were made by unpaired, two-tailed Student's t-test. Analysis of covariance was used to adjust for differences in age between diabetic and control subjects and for differences in age, duration of diabetes, and presence of proteinuria amongst the diabetic subjects. The relation of IGF-1 levels to the clinical variables was determined by linear regression analysis and expressed as the correlation coefficient. Significance was taken at the 0.05 level. All group values are expressed as mean ± SE unless otherwise stated. Body mass index (BMI) was calculated as weight/height (kg/m^2).

Results

The mean area under the hGH curve was higher in the 4 patients admitted for 24-h profiles than in the 4 normal subjects (284 ± 24 vs 98.3 ± 28.3 mU $l^{-1}h^{-1}$; $p<0.05$). The mean coefficient of variation (CV) for plasma hGH was 84 % in the diabetic subjects and 86 % in the normal subjects; the mean CV of the IGF-1 values was 19.7 % in the diabetic stubjects and 14.9 % in the normal subjects.

Individual values of IGF-1 in the normal subjects and the diabetic subjects divided according to retinopathy

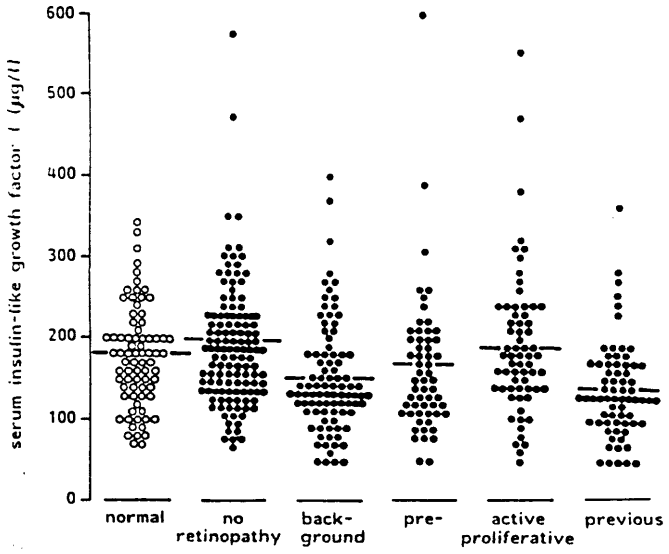


Figure 1. Individual serum IGF-1 values for normal subjects and patients divided according to retinopathy type. The bar represents the mean for each group

appearance are shown in Figure 1. No values were outside the normal range or in the acromegalic range (>700 μg/l). The three individuals in the proliferative subgroup who had the highest levels (>400 μg/l) were noted to be young diabetic patients (mean age 27 years) with very active and extensive new vessel formation.

The mean IGF-1 level in the control and diabetic subjects did not differ significantly (177 ± 7.4 vs 168.4 ± 3.9 μg/l; NS). There was no significant difference in mean IGF-1 value between the diabetic patients with no retinopathy and those with active proliferative change (198.7 ± 8.8 vs 190.5 ± 11 μg/l; NS).

Diabetic patients without retinopathy and those with active proliferative retinopathy had significantly higher mean IGF-1 levels than the normal subjects. This difference persisted after adjusting for age differences (164.9 ± 9.07 (controls) vs 192.7 ± 8.3 (no retinopathy) and 191 ± 9.5 (proliferative retinopathy) μg/l; $p<0.05$).

These two subgroups also had higher mean IGF-1 values than the other diabetic groups. However, after adjusting for differences in age, duration of diabetes, and the presence of proteinuria, there were no significant differences between the patient subgroups, apart from the inactive previously proliferative group which showed significantly lower mean values than the other subgroups (151.8 ± 11.5 μg/l; $p<0.05$). These results are summarized in Table 2.

A significant negative correlation was noted between IGF-1 and age ($r=-0.49$, $p<0.001$ (normal controls); $r=-0.23$, $p<0.05$ (patients)). The presence of proteinuria was significantly associated with IGF-1 level ($r=0.42$, $p<0.001$). Proteinuria was absent in the no retinopathy subgroup and most frequently present in the preproliferative and active proliferative patients (Table 1).

Weaker associations were found between IGF-1 and BMI and HbA_1; these are summarized in Table 3. There was no significant correlation between IGF-1 and sex, duration of diabetes, treatment with insulin, or random blood glucose.

Discussion

IGF-1 values were similar in diabetic and normal subjects in accordance with previous studies employing radioimmunoassay methods.[9,19,20] There was little diurnal variation in IGF-1 level in normal and diabetic subjects, allowing single clinic samples to be used.

We found no significant difference in mean serum IGF-1 concentration between the diabetic patients without retinopathy and those with active proliferative changes. This would argue against the hypothesis that serum IGF-1 concentration is causally related to the development of proliferative retinopathy. Furthermore, there was no significant difference in mean IGF-1 values between patients with preproliferative retinopathy (in whom extensive vascular occlusion was present on

Table 2. Serum IGF-1 concentration (μg/l) in the control and diabetic subjects studied

Group	IGF-1	Age adjusted IGF-1	Age, duration, proteinuria adjusted IGF-1[a]
Control	177.0 ± 7.4	164 ± 9.1	n/a
All diabetic	168 ± 3.9	172 ± 9.1	n/a
No retinopathy	198.7 ± 8.8[b]	192.7 ± 8.3[c]	205.1 ± 16.3
Background retinopathy	148.7 ± 6.9	153.4 ± 8.2	164.5 ± 11.7
Pre-proliferative retinopathy	166.7 ± 12.6	177.8 ± 10.2	165.2 ± 13.1
Active proliferative retinopathy	190.5 ± 11.0[b]	191.0 ± 9.5[c]	181.2 ± 14.2
Previous proliferative retinopathy	137.2 ± 6.7	148.4 ± 9.1	151.8 ± 11.5[b]

[a]Adjusted for differences within diabetic group.
Mean ± SE
[b]$p<0.05$, compared with other diabetic subgroups.
[c]$p<0.05$, compared with control subjects.

Table 3. Correlation of IGF-1 with clinical variables

Association	Group	p	r	Relation
IGF-1 and age	Control	0.001	-0.49	IGF-1 = 266–2.50 × age
	Diabetic	0.05	-0.23	IGF-1 = 247–1.21 × age
IGF-1 and sex	Control	NS		
	Diabetic	NS		
IGF-1 and BMI	Control	NS		
	Diabetic	0.05	-0.22	IGF-1 = 332–4.99 × BMI
IGF-1 and HbA_1	Diabetic	0.05	-0.20	IGF-1 = 108–8.76 × HbA_1
IGF-1 and duration diabetes	Diabetic	NS		
IGF-1 and insulin treatment	Diabetic	NS		
IGF-1 and proteinuria	Diabetic	0.001	+0.42	IGF-1 = 138+44.8 × proteinuria
IGF-1 and random blood glucose	Diabetic	NS		

Age (years), BMI (kg/m^2), HbA_1 (%), proteinuria (present = 1).

fluorescein angiography) and diabetic patients with no retinopathy. These observations are consistent with other studies[9,11,16] in which diabetic patients with and without retinopathy had similar IGF-1 levels.

In this study, there was a highly significant correlation between serum IGF-1 concentration and the presence of proteinuria, suggesting that nephropathy before overt renal failure may increase IGF-1 levels. The kidney is an important site of IGF-1 degradation[21] and an increase in IGF-1 level would not be unexpected, analogous to the rise in serum insulin during the development of renal failure.[22]

Ashton *et al.*[14] reported that mean somatomedin activity as measured by a bioassay method, is higher in insulin-dependent diabetic patients with retinopathy than in non-diabetic subjects. As in the study of Merimee *et al.*,[15] there is no information on the presence of proteinuria, which was significantly more severe and more frequent in our patients with proliferative retinopathy and which is the likely explanation for elevated levels of somatomedin in this group as compared with control subjects.

A subgroup of patients with rapidly accelerating retinopathy has been reported to have particularly high serum IGF-1 levels.[15] We noted that in the proliferative group, IGF-1 values were highest in young diabetic patients with very active retinopathy. As in other studies,[13] serum IGF-1 was negatively associated with age, so that the younger age of these patients may have contributed to their higher IGF-1 values.

Interestingly, patients with inactive previously proliferative retinopathy had the lowest mean IGF-1 levels, significantly lower than the other patient groups. Whether this was the result of laser treatment or whether these patients also had lower IGF-1 values when they had active proliferative retinopathy can only be resolved by prospective studies.

Local concentration of IGF-1 may be more relevant than circulating levels when its role in diabetic retinopathy is being considered, since retinal endothelial cells are capable of producing this and other growth factors.[23] The finding of elevated levels in the vitreous of patients with advanced proliferative retinopathy is therefore of great interest,[24] though it is not established whether this is produced locally in the proliferating retina or leaks out of retinal vessels.

In summary, serum IGF-1 concentration corrected for differences in age and presence of proteinuria was found to be similar in patients with varying grades of retinopathy apart from the inactive laser treated group who continued to show lower levels than other diabetic patients. Our results do not exclude a temporary rise in IGF-1 levels during an early active phase which subsequently returns to normal. Prospective studies to examine this possibility are currently in progress.

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Continuous subcutaneous octreotide infusion markedly suppresses IGF-I levels whilst only partially suppressing GH secretion in diabetics with retinopathy

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Abstract. The response to GH releasing hormone (GHRH 1–29) and 24-h serum GH and IGF-I levels were measured in 9 insulin-dependent diabetics with retinopathy and 6 normal volunteers before and after different treatment regimens with octreotide, a long-acting somatostatin analogue. Octreotide, 50 μg by sc injection, completely suppressed GHRH-stimulated GH release in both groups. Thrice daily sc injections for up to 20 weeks were associated with variable plasma octreotide levels and failed completely to suppress GH secretion in either the patients or the normal controls. Three days of continuous sc pump infusion (500 μg/24-h) resulted in consistently high plasma octreotide levels and completely suppressed 24-h GH in 4 normal subjects, whilst treatment for up to 16 weeks only partially suppressed GH levels in 6 patients (AUC $mU \cdot l^{-1} \cdot h^{-1}$; 209 ± 81 vs 121 ± 82; $P = 0.01$). Mean ± SD IGF-I levels (μg/l) in the patients (but not controls) were suppressed into the hypopituitary range by median 6 weeks (range 2–16) pump administration (203 ± 62 vs 60 ± 25; $P = 0.02$). Pump treatment achieved total GH suppression in normal subjects; diabetics with retinopathy seem more resistant to the GH suppressing effects of the drug. However, the reduction of serum IGF-I with prolonged treatment may be of clinical value in arresting the progress of diabetic retinopathy.

Inhibition of GH secretion by hypophysectomy or yttrium implantation was an effective treatment of diabetic retinopathy in the past (Lundbæk et al. 1969; Kohner et al. 1976; Sharp et al. 1987). Although this has now been superceded by laser photocoagulation and vitrectomy, the amount of photocoagulation required often results in visual field defects and problems with night vision, and in a small proportion of patients, retinopathy remains active. Pharmacological suppression of GH in these circumstances might be a useful adjunct to photocoagulation as the benefits of hypophysectomy are related to the degree of GH deficiency achieved (Wright et al. 1969; Adams et al. 1974).

Octreotide (SMS 201-995, Sandostatin®, Sandoz, Basel), an analogue of somatostatin, is a highly potent GH inhibitor (Marbach et al. 1985) with a slow plasma clearance and lack of rebound GH release after subcutaneous injection (Del Pozo et al. 1986). Because its duration of GH inhibition in normal volunteers and diabetic patients is 3–6 h (Davies et al. 1986a,b; Lamberts 1986), multiple daily injections are required to maintain 24-h GH suppression.

We report here the results of a feasability study investigating the possibility of long-term treatment with octreotide given as a continuous sc infusion via a portable pump or as thrice daily sc injections. We studied diabetics with proliferative or preproliferative retinopathy and a group of normal volunteers.

Patients and Methods

Nine patients with insulin-dependent diabetes and 6 age-matched normal control subjects were studied. Patients were selected if they had active proliferative diabetic retinopathy that had not responded adequately to photocoagulation (8 patients) or severe preproliferative changes in both eyes (1 patient) equivalent to level 40/40 on the Wisconsin grading system for diabetic retinopathy (Klein et al. 1984). The clinical details of the patients are shown in Table 1. Informed written consent was obtained from all study subjects and the protocol approved by the Ethical Committee of the Royal Postgraduate Medical School and Hammersmith Hospital.

Studies of stimulated GH release were performed in all subjects. Serum GH levels were measured for 15 min before and every 15 min for 90 min after a bolus of GHRH 1–29 (Bachem), 100 μg iv dissolved in 10 ml of acidified saline and given over 2 min. This was repeated on a separate day after pretreatment with 50 μg of octreotide sc given 30 min prior to the GHRH injection.

All subjects were admitted to the Metabolic Unit for studies. They were also examined ophthalmologically including colour retinal stereophotography and fluorescein angiography. Blood was taken for measurement of HbA_1. Two baseline 24-h studies were performed during which blood was sampled at hourly intervals for serum GH and 6 hourly for measurement of serum IGF-I. Subjects remained ambulant throughout the sampling day and retired to bed at about 22.00 h.

In the first part of the study, 4 patients (Patients No. 1–4, Table 1) and 6 controls received octreotide, 50 μg, by sc injection at 06.00, 14.00 and 22.00 h for 3 days and the 24-h blood sampling was repeated.

In order to test the feasability of long-term treatment with this drug, the above 4 patients were then instructed on self administration and continued treatment at home (median total duration of treatment: 14 weeks; range 8–20). The daily dose of octreotide was increased at 2 weekly intervals from 100 μg × 3, to 200 μg × 3, to 500 μg × 3. All patients tolerated 200 μg × 3 (2–6 weeks), but only 3 patients agreed to receive the higher dose of 500 μg × 3 (8–16 weeks).

Treatment was stopped because of intolerable gastrointestinal symptoms, particularly abdominal discomfort and steatorrhea. Blood sampling was performed during the last 24-h of each treatment regimen.

Six patients (Patients No. 3, 5–9, Table 1) and 4 of the control subjects participated in the second part of the study investigating possible advantages of continuous sc pump delivery. Patient No. 3 entered this part of the study 6 months after finishing the earlier section. After the initial baseline measurements, pump treatment (Graseby) was begun at an infusion rate of 500 μg octreotide/24-h sc and continued for 3 days at which time the GH profile was repeated.

A further 24-h study was performed in subjects who appeared to show GH suppression during which blood samples were taken at hourly intervals during the day and at 20-min intervals from 23.00 to 08.00 h. Patients continued pump treatment for 2–16 weeks (median 6 and were restudied during the last 24-h of treatment.

Assays

Serum was separated and stored at −20°C until assayed. GH was measured with a specific double-antibody RIA by an automated system (Kemtek, Burgess Hill, Sussex, UK) using the WHO International Reference preparation for human growth hormone (66/217, 350 mU = 175 μg) as

Table 1.
Clinical details of patients.

Patient M/F	VA		Age (years)	Duration (years)	BMI	HbA_1 (%)		Creatinine (μmol/l)		Proteinuria (g/l)
	R	L				(a)	(b)	(a)	(b)	
1 M	6/36	6/5	27	19	28.6	12.1	12.2	106	117	3
2 F	6/12	6/36	22	18	27.1	10.5	12.0	106	80	2
3 F	6/6	6/9	45	33	23.1	10.3	10.9	71	53	0
4 F	6/9	6/9	31	20	25.4	9.4	9.1	72	80	1
5 M	6/18	6/9	36	17	21.9	9.3	9.1	88	106	2
6 F	HM	6/18	34	18	26.1	7.6	5.2	71	62	2
7 F	6/18	CF	24	17	18.7	9.7	9.6	63	62	2
8 M	6/6	6/6	28	25	24.8	11.8	9.8	88	97	2
9 M	6/9	6/6	26	23	20.9	8.1	6.2	96	80	2

Abbrevations: M/F: male/female; R/L: right/left eye; VA: visual acuity at entry; HM: hand movements; CF: counting fingers; BMI: body mass index (weight/height^2). HbA_1 and serum creatinine results are shown (a) before and (b) after octreotide treatment.

standard (Burrin et al. 1985). The within and between assay coefficients of variation were 4.7 and 11.4% at a GH concentration of 14.3 mU/l. The detection limit of the assay was 0.5 mU/l. Serum IGF-I was determined after acid ethanol extraction (Daughaday et al. 1980) using monoclonal antibody 3D 1/2/1 kindly donated by Dr Baxter (Sydney, Australia) through the National Hormone and Pituitary Program. Samples with values at the lower limit of detection by this assay (100 μg/l) were reassayed using polyclonal antibody UB 286 (kindly donated by Drs L. Underwood and J. J. Wyck, University of North Carolina, Chapel Hill, NC). The limit of detection of this assay was 5 μg/l.

Both assays show excellent correlation for IGF-I values in the 100–300 μg/l range (Burrin et al. 1987), but the monoclonal assay is unsuitable for values in the hypopituitary range. The intra- and inter-assay coefficients of variation of the two assays did not differ significantly. At an IGF-I concentration of 120 μg/l, they were 5.3 and 10.2% respectively, normal range: 100–350 μg/l.

Octreotide was donated by Sandoz, Basel, Switzerland, as was octreotide antibody and ^{125}I-labelled antigen for RIA. Total glycosylated hemoglobin was measured by agar gel electrophoresis and is expressed as percentage of total hemoglobin in the glycosylated form (normal range: 5.5–8.5%).

Analysis

The 24-h GH secretion was expressed as the area under the GH curve (AUC) calculated by the trapezoidal rule. Results were analysed non-parametrically; comparisons before and after treatment within the same group were made using the Wilcoxon test for paired data. The Mann-Whitney test was used to compare results between groups. Significance was taken as $P < 0.05$. Body mass index is calculated as weight (kg)/height (m)^2. Duration of treatment is expressed as the median value together with the range of values. All other values in the text are expressed as mean ± SD.

Results

Growth hormone concentration

The response to GHRH was similar in patients and control subjects. Octreotide, 50 μg sc, completely inhibited GHRH-induced GH release in the patient group and in the controls (Fig. 1).

Baseline 24-h GH secretion expressed as mean AUC ± SD (mU · l^{-1} · h^{-1}) was increased in the patients compared with the controls (285 ± 99 patients vs 154 ± 72 controls; $P < 0.05$). There was no significant GH suppression after 3 days treatment with thrice daily octreotide injections in either the control subjects: baseline 154 ± 72 vs 94 ± 69; $P > 0.05$) or in the patients: baseline 285 ± 99 vs 213 ± 74.

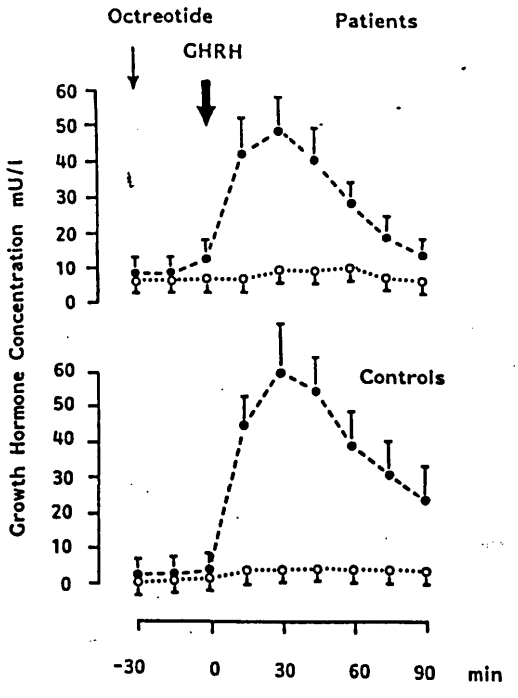


Fig. 1.
Serum GH response to a bolus of GHRH, 100 μg iv, before and after pretreatment with octreotide 50 μg sc in diabetic patients (N = 4) (upper panel) and normal volunteers (N = 6) (lower panel).

Continued injection treatment for a total of 8–20 weeks failed to suppress 24-h GH secretion in the patients; mean AUC 205 ± 28 (200 μg × 3 for 2–6 weeks); 234 ± 83 (500 μg × 3 for 8–16 weeks). By contrast, continuous sc pump infusion for 3 days completely suppressed GH secretion in 4 control subjects confirmed by 20-min sampling overnight (all GH values <0.5 mU/l). The 24-h GH secretion was reduced but not totally suppressed after 6 weeks (range 2–16 weeks) pump treatment in the 6 patients; baseline 209 ± 81 vs 121 ± 82 after treatment; $P = 0.01$ (Table 2).

The pattern of GH secretion during octreotide treatment in the patients is illustrated in Figs. 2 and 3.

Plasma octreotide concentration

Drug levels were variable after injection treatment compared with consistently high values (greater than 2.5 μg/l) achieved with pump treatment.

Table 2.
Changes in area under 24 h GH curve (AUC) and serum IGF-I concentration in patients treated with octreotide 500 µg/24 h by continuous sc infusion.

Patient	Duration treatment (weeks)	AUC (mU · l^{-1} · h^{-1})		IGF-I (µg/l)	
		Baseline	After treatment	Baseline	After treatment
1	6	258	201	142	70
2	6	173	80	174	38
3	16	129	58	198	33
4	2	350	243	312	55
5	8	180	40	235	65
6	2	165	103	159	102
Mean	6.7	209.2	120.8*	203.3	60.5*
SD	5.2	80.9	82.2	62.3	25.0

* $P < 0.05$ compared with baseline value.

Plasma trough levels of octreotide immunoreactivity greater than about 1 µg/l were sufficient to suppress serum GH in the control subjects. Breakthrough GH secretion in the control subjects occurred on injection treatment when drug levels fell below this level. However, despite high circulating octreotide levels during injection or pump treatment, spontaneous GH peaks continued to occur in the diabetic patients.

IGF-I concentration

Treatment in the patient group by continuous sc pump infusion for 3 days resulted in a significant fall in mean IGF-I (203 ± 62 vs 102 ± 33 µg/l; $P < 0.05$). Continued pump treatment for 6 weeks (range 2–16 weeks) resulted in a much greater reduction in mean IGF-I levels (203 ± 62 vs 60 ± 25; $P = 0.02$) than in 14 weeks (range 8–20 weeks) of octreotide by thrice daily injections: 219 ± 55 (ba-

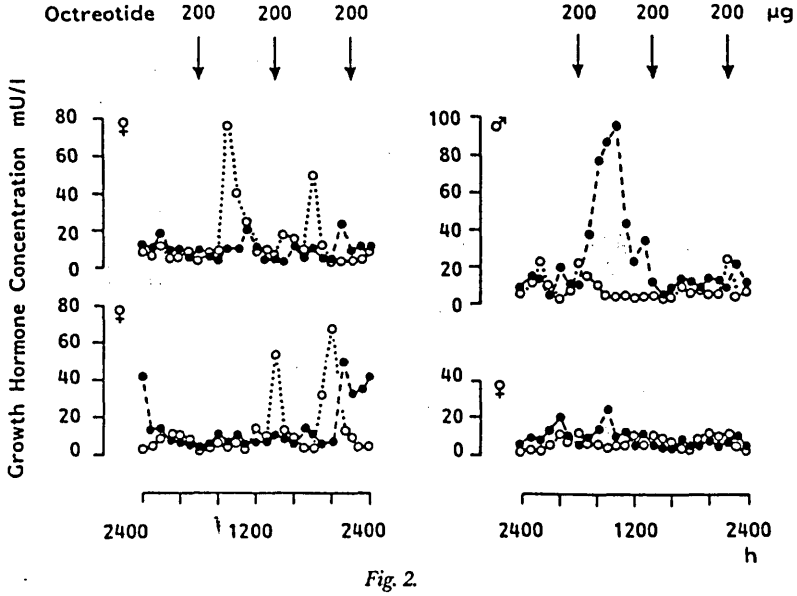


Fig. 2.
Twenty-four hour serum GH profiles in 4 diabetic patients before (closed circles) and after (open circles) octreotide, 200 µg × 3 daily by sc injection for 2–6 weeks.

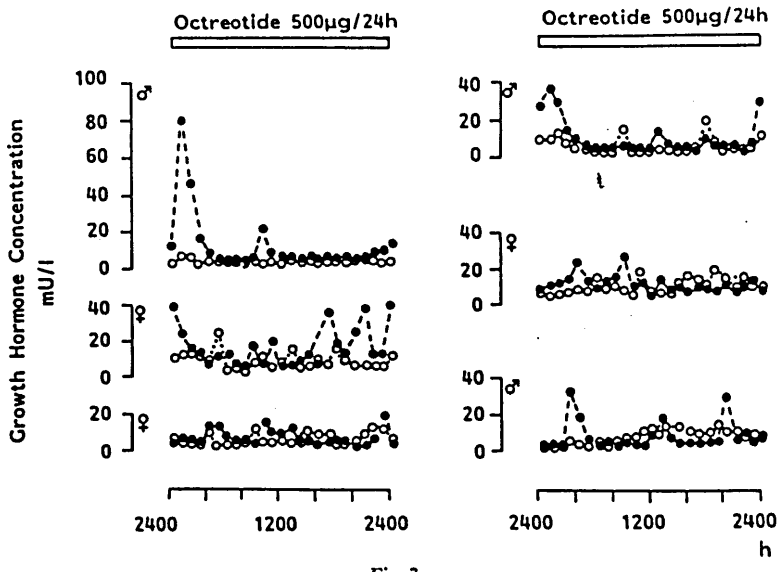


Fig. 3.

Twenty-four hour serum GH profiles in 6 diabetic patients before (closed circles) and after (open circles) 6 weeks (range 2–16 weeks) octreotide 500 µg/24 h by continuous sc pump infusion.

seline); 195 ± 73 (200 µg × 3); $P > 0.05$; 180 ± 75 (500 µg × 3), $P = 0.05$.

IGF-I values in the patients after pump treatment were in the same range as 14 successfully pituitary-ablated diabetic patients (Fig. 4).

By contrast, mean IGF concentration in the control subjects was not significantly different compared with baseline values after 3 days pump (N = 4) or 3 days injection treatment (N = 6) (211 ± 57 vs 128 ± 45 (pump), $P = 0.1$; 217 ± 61 vs 125 ± 48 (injection), $P = 0.06$).

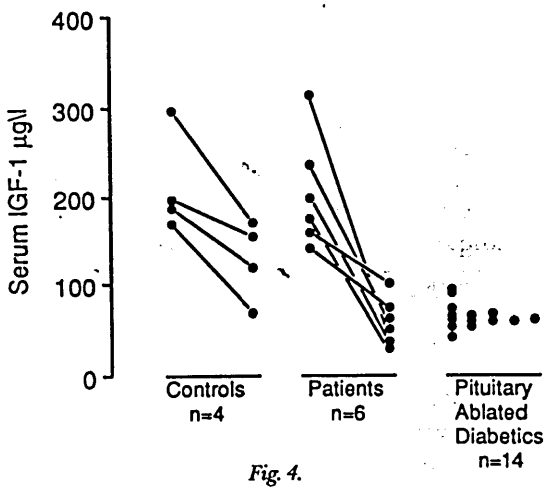


Fig. 4.

Mean serum IGF-I before and after octreotide by continuous sc pump infusion in patients (after 2–16 weeks) and normal volunteers (3 days). IGF-I values in a group of pituitary-ablated diabetics are shown for comparison.

Ophthalmic assessment

During intermittent injection treatment, visual acuity deterioriated by at least 2 lines in 3 of 4 patients, whereas there was no change (5 patients) or improvement (1 patient) in vision in patients receiving pump treatment. Treatment did not prevent recurrent retinal hemorrhages and further laser treatment was necessary in all patients. One patient who received 2 months of intermittent injection treatment subsequently underwent vitrectomy 14 months after cessation of octreotide treatment. There was no progression of preproliferative retinal changes (1 patient) during octreotide treatment.

Adverse effects

All patients and control subjects experienced diffuse abdominal discomfort and passed pale offensive motions during treatment, particularly at doses in excess of 100 µg three times daily. There was no relation of abdominal symptoms to the presence of autonomic neuropathy. Injections were painful although surprisingly, there was little local discom-

fort from continuous pump infusion. There was no tendency for gastrointerstinal symptoms to improve with continued therapy. One patient lost 7 kg in weight after 3 weeks of pump treatment and another experienced transient amenorrhea. Daily insulin requirements fell by 50% with no significant change in mean HbA_1 during the study period (Table 1). There was an increased incidence of hypoglycemia which tended to be severe and slow to respond to oral glucose. No deterioriation of renal function as reflected by serum creatinine and degree of proteinuria, occurred during treatment.

Discussion

In this study, pump infusion at a dose of 500 μg/24 h was superior to intermittent injections at doses up to 500 μg eight hourly, resulting in consistently high serum octreotide levels and greater suppression of serum GH and IGF-I levels. A similar finding was recently reported in a study comparing pump (100 μg/24 h) and injection treatment in patients with acromegaly (Christensen et al. 1987). In our patients, GH levels were escaping 2–4 hours after injections, even at times when drug levels were elevated (Kutz et al. 1986). In addition, spontaneous GH peaks were observed in the diabetics (but not in control subjects) during pump infusion, again when drug levels were high. This persistence of diurnal GH secretion in the presence of octreotide is in contrast to the marked blunting by the drug of GHRH-stimulated GH release and suggests that central regulatory mechanisms controlling basal GH, probably in the hypothalamus, are relatively resistant to somatostatin in these patients. This would be consistent with the failure of glucose-mediated GH suppression (Sharp et al. 1984; Press et al. 1984) and increased GH responses to provocative stimuli (Passa et al. 1974; Kaneko et al 1985) in patients with diabetic retinopathy.

Interestingly, although pump infusion did not totally suppress GH secretion in the patients, the levels of IGF-I achieved were very low, in the same range as successfully pituitary-ablated diabetic patients. This could imply that octreotide acts peripherally (possibly on liver receptors) to inhibit IGF-I production. Prolonged treatment may result in low IGF-I levels as a reflection of the patients' poor nutrition during treatment (Phillips 1986) although only one patient lost a large amount of weight.

The combination of higher basal GH secretion yet similar IGF-I levels in the patient group compared with the controls suggests that IGF-I production was already impaired in the patients before treatment. This is consistent with reports of impaired IGF-I generation in poorly controlled diabetes (Lanes et al. 1985; Amiel et al. 1984). IGF production may therefore be particularly susceptible to somatostatin in patients with diabetes although the duration of treatment in the normal subjects may not have been long enough to achieve similar IGF-I suppression.

Both circulating and local levels of IGF-I may be important in the development of diabetic retinopathy (Ashton et al. 1983; Merimee et al. 1983; Grant et al. 1986) and if so, the suppression of IGF-I concentration achieved by pump treatment might be expected to arrest the progression of the eye changes.

Despite a decrease in IGF-I levels into the hypopituitary range, there was no discernible effect on retinopathy appearance and retinal hemorrhages were not prevented by treatment. This probably reflects the advanced stage of the retinopathy. Indeed, pituitary ablation is of little value in patients with advanced fibrovascular proliferation (Sharp et al. 1987). Interestingly, there had been no progression of retinopathy in the one patient with preproliferative changes during two months of treatment.

Treatment with octreotide was associated with persistent unpleasant gastrointestinal symptoms in all subjects which were relieved only after discontinuation of therapy. These effects are likely to be secondary to gut hormone suppression by octreotide (Williams et al. 1986). Similar symptoms were reported in normal volunteers (Davies et al. 1986a) and diabetics (Davies et al. 1986b) receiving 50 μg octreotide twice daily.

Severe hypoglycemia was also a problem in the patient group despite a 50% reduction in total daily insulin dosage and was poorly responsive to oral glucose. Octreotide suppresses glucagon levels for up to 6 hours (Davies et al. 1986a) and also inhibits the GH response to hypoglycemia (Lightman et al. 1986).

Although post-prandial hyperglycemia is improved by single injections of octreotide (Spinas et

al. 1985; Serrano-Rios et al. 1986), we found no overall improvement in glycemic control after treatment.

Another potential problem with octreotide is attenuation of its inhibiting effects with chronic treatment. This has been observed in non-diabetic animals (Lamberts et al. 1987) but, at least in acromegalic patients, does not seem to occur despite up to 24 months of treatment (Dieguez et al. 1988). In this study, IGF-I levels remained suppressed for up to 16 weeks of pump treatment.

In summary, we and others (Davies et al. 1986b; Plewe et al. 1987) have not been able to achieve complete 24-h GH suppression with this drug in diabetic patients. More detailed dose-response studies are required to assess the apparent resistance to the GH suppressing effects of somatostatin in these patients. Long-term studies are currently in progress to see if the partial GH suppression and marked IGF-I reductions achieved will be sufficient to arrest the progression of early preproliferative retinopathy.

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Failure to suppress GH secretion after 2 weeks treatment with atropine or propanthelene in diabetics with proliferative retinopathy

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Abstract. Complete suppression of GH secretion may halt the development of retinal new vessels in patients with diabetic proliferative retinopathy. We have investigated the effectiveness of two cholinergic antagonists, atropine and propanthelene given orally for 2 weeks, in suppressing 24-h GH and IGF-I levels. Seven insulin-dependent diabetics (3 males, 4 females; aged 22–34 years) with active proliferative retinopathy and 6 matched non-diabetic normal subjects were studied in random order with at least 2 weeks between treatments. Suppression of GHRH-induced GH release was demonstrated in both groups of subjects. Twenty-four hour GH secretion was not, however, suppressed in either the patient group (mean area under the GH curve $mU \cdot l^{-1} \cdot h^{-1} \pm SD$; baseline: 251 ± 108.7; after atropine: 174 ± 106.9; after propanthelene: 180 ± 72.4; $P > 0.05$) or in the control group (baseline: 103 ± 53.1; after atropine: 73 ± 83.6; after propanthelene: 122 ± 71.6; $P > 0.05$). GH release at times of hypoglycemia was not suppressed. Mean IGF-I concentration was not significantly reduced. Two subjects (one patient and one control) could not tolerate atropine for more than one week. We conclude that repeated doses of atropine and propanthelene do not achieve complete 24-h GH suppression and are associated with a high incidence of unpleasant adverse reactions.

Several studies have demonstrated that hypophysectomy or pituitary ablation with ^{90}Y can arrest or reverse the progression of proliferative retinopathy and that this beneficial effect persists at 10 year follow-up (Lundbæk et al. 1969; Kohner et al. 1976; Sharp et al. 1987). The degree of GH deficiency achieved correlated with the beneficial effect on retinopathy (Wright et al. 1969; Adams et al. 1974). More recently, elevated circulating and vitreous concentrations of GH dependent insulin-like growth factor I have been found in diabetics with rapidly progressive proliferative retinopathy (Merimee et al. 1983; Grant et al. 1986).

These findings suggest a role for GH directly or via IGF-I in promoting the development of proliferative retinopathy. Selective GH suppression, if equally effective, would be preferable to pituitary ablation in patients with retinal new vessels persisting despite laser treatment.

Cholinergic muscarinic receptor antagonists are potent and selective inhibitors of GH release under a variety of conditions in both normal and diabetic subjects (Delitala et al. 1983; Casanueva et al. 1984; Davis & Davis 1986; Page et al. 1987). The aim of this study was to assess the feasability of using two such cholinergic antagonists, atropine and propanthelene, to suppress GH in patients with active proliferative retinopathy despite repeated laser therapy.

Patients and Methods

Seven patients with insulin-dependent diabetes and active proliferative retinopathy despite repeated laser therapy were selected for study. Serum creatinine was

Table 1.
Clinical characteristics of patients.

Patient	Sex	Age (years)	Body mass index	Duration of diabetes (years)	Proteinuria (g/l)	HbA_1 (%)
1	M	27	28.6	19	1	12.1
2	F	22	27.1	18	0	10.5
3	F	45	23.1	33	0	10.3
4	F	31	25.4	20	0	9.4
5	M	36	21.9	17	0	9.3
6	F	34	26.1	18	0	7.6
7	F	24	18.7	17	0	9.7

within the normal range although one patient had proteinuria. The clinical details are shown in Table 1. Six non-diabetic normal subjects (4 males, 2 females: aged 24–33 years) were also investigated by the same procedure. The study was approved by the Ethical Committee of the Royal Postgraduate Medical School and Hammersmith Hospital.

Subjects were admitted to the Metabolic Unit for 24-h studies of GH secretion after an overnight fast. An indwelling catheter was inserted into a forearm vein for the purpose of blood sampling. Blood was drawn without disturbing the subjects for measurement of serum GH, IGF-I and plasma glucose of hourly (06.00–24.00) or 2-hourly intervals (24.00–06.00) with at least 21 samples over 24-h. Subjects were encouraged to remain ambulant throughout the day and retired to bed at 22.30 h.

Baseline 24-h sampling was performed on two occasions separated by at least 2 weeks. Patients and control subjects then received, on separate occasions, in randomised order, oral atropine sulphate 1.2 mg to be taken at 4-h intervals starting at 06.00 h, and oral propanthelene bromide 30 mg taken from 06.00 h at 8-h intervals. After 2 weeks of treatment, 24-h GH and IGF-I profiles were repeated. The analysis was performed masked to the subjects' current treatment.

Studies of stimulated GH release were performed at the end of the 24-h baseline studies. Blood samples were drawn at 15-min intervals for 30 min before and 90 min after a bolus of GH-releasing hormone 1–29 NH_2 (GHRH) 1 μg/kg iv dissolved in 10 ml of acidified saline and given over 2 min. This test was repeated after pretreatment with atropine 1.2 mg im at −15 min or propanthelene 45 mg po at −30 min.

Assays

Serum was stored at −20°C until assayed. Serum GH was measured with a specific double-antibody radioimmunoassay by an automated system (Kemtek, Burgess Hill, Sussex, UK) using the WHO International Reference Preparation for human growth hormone (66/217, 350 mU = 175 μg) as standard (Burrin et al. 1985). The within and between assay coefficients of variation were 4.7 and 11.4% at a GH concentration of 14 mU/l. The detection limit of the assay was 0.5 mU/l. Samples from a particular subject before and after a test drug were analysed on the same assay.

Serum IGF-I was measured by double-antibody RIA using the monoclonal antiserum 3D 1/2/1 supplied through the National Hormone and Pituitary Program. Before measurement, samples were acid ethanol stripped to separate IGF-I from its binding protein (Daughaday et al. 1980). At an IGF-I concentration of 200 μg/l, the intra- and inter-assay coefficients of variation were 5.3 and 10.2%, respectively; normal range: 100–350 μg/l. HbA_1 was measured by agar gel electrophoresis; normal range: 5–8%. Plasma glucose was measured by the glucose oxidase method using the Beckman Glucose Analyser (Beckman Instruments, Palo Alto, CA).

Analysis

The 24-h GH secretion was measured as the area under the GH curve calculated by the trapezoidal rule. Results were analysed non-parametrically; comparisons before and after treatments within the same group were made by the Wilcoxon test for paired data and between groups by the Mann-Whitney test. $P < 0.05$ was taken as the level of significance. Body mass index was calculated as wt (kg)/height $(m)^2$. Values in the text are expressed as mean ± SD.

Results

Atropine, 1.2 mg by im injection, and propanthelene, 45 mg po, were effective in suppressing the GH response to GHRH in the patient and

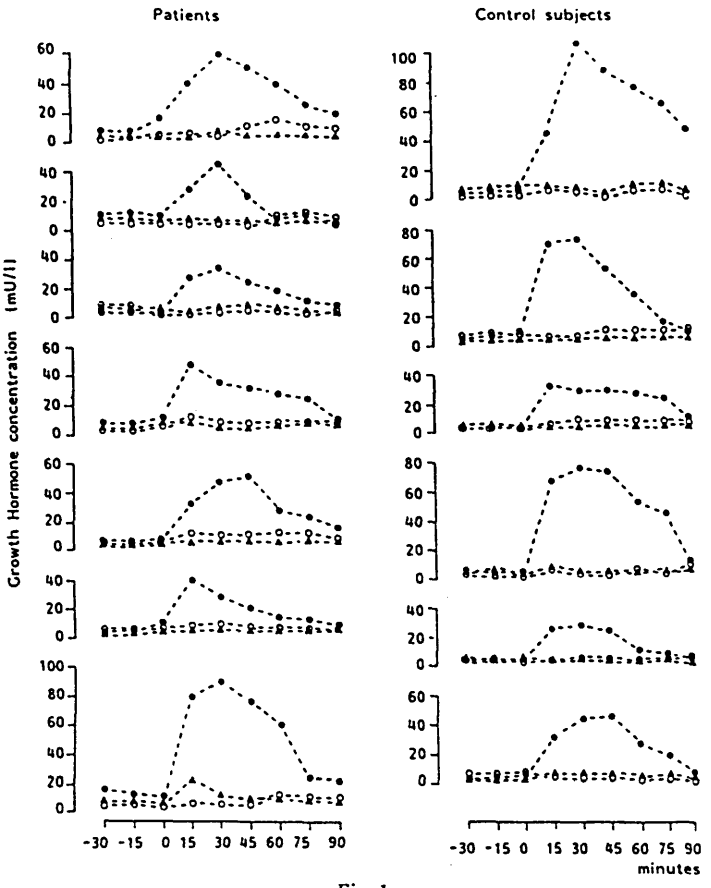


Fig. 1.
Serum GH response to a bolus of 1 μg/kg GHRH 1-29 at time 0; GHRH alone (closed circles), after pretreatment with atropine 1.2 mg im at −15 min (triangles), and after pretreatment with propanthelene 45 mg po at −30 min (open circles) in 6 normal subjects and 7 diabetic patients.

control groups (Fig. 1). Table 2 gives the results of the 24-h studies. Baseline values taken on two control 24-h periods did not differ significantly in either control subjects or patients.

All subjects experienced unpleasant dryness of mouth, difficulty of swallowing, blurring of vision and tachycardia. Two subjects (one patient and one control) could not tolerate oral atropine for more than a week; studies were performed at one week on them.

After treatment with oral atropine, the mean area under the GH curve (AUC) ± SD in the control subjects was reduced from 103 ± 53.1 (baseline) to 73 ± 83.57 $mU \cdot l^{-1} \cdot h^{-1}$, but this was not statistically significant ($P > 0.05$). Mean AUC after propanthelene treatment was 122 ± 71.6 $mU \cdot l^{-1} \cdot h^{-1}$, which was not significantly different from baseline values or from results after atropine ($P > 0.05$). There was no significant difference in numbers of GH peaks during either treatment compared to baseline.

Baseline 24-h GH secretion was greater in the 7 patients compared with the control subjects, although this did not achieve statistical significance (251 ± 108.7 vs 103 ± 53.1 $mU \cdot l^{-1} \cdot h^{-1}$; $P > 0.05$).

Mean AUC compared with baseline values was not significantly reduced by either atropine (251 ± 108.7 vs 174 ± 106.9; $P > 0.05$) or propanthelene (251 ± 108.7 vs 180 ± 72.4; $P > 0.05$) in the patient group. The number of GH peaks during treatment compared with baseline values was not significantly reduced after atropine (4.14 ± 1.2 vs 2.7 ± 1.1; $P > 0.05$) or after propanthelene (4.14 ± 1.2 vs 2.86 ± 1.1; $P > 0.05$).

The pattern of GH secretion is illustrated in Fig. 2 (control subjects) and Fig. 3 (patients). GH

Table 2.
Area under the 24-h GH curve (AUC) mU · l^{-1} · h^{-1} before and after atropine and propanthelene.

	Baseline*	Atropine 1.2 mg × 4 h	Propanthelene 30 mg × 6 h
Control subjects			
1	47	20	71
2**	113	20	67
3	81	63	232
4	167	50	146
5	161	240	167
6	49	45	49
Mean ± SD AUC	103 ± 53.1	73 ± 83.57	122 ± 71.6
Patients			
1	428	161	145
2	247	145	236
3	138	68	110
4	258	104	107
5**	206	126	140
6	350	390	225
7	129	224	295
Mean ± SD AUC	251 ± 108.7	174 ± 106.9	180 ± 72.38

* Mean of two control nights.
** Indicates subjects who only tolerated atropine for 1 week.

peaks associated with hypoglycemia (plasma glucose < 2.2 mmol/l) were not suppressed by atropine or propanthelene as illustrated by 4 representative patients shown in Fig. 4. There was no difference in frequency of hypoglycemic episodes during baseline measurements or during treatment with either drug.

Mean 24-h serum IGF-I (μg/l) was not significantly reduced after either drug in the normal subjects (baseline: 203 ± 47.9; after atropine: 166 ± 34.3; after propanthelene: 214 ± 57.4; $P > 0.05$) or in the patient group (baseline: 279 ± 136.4; after atropine: 195 ± 36.6; after propanthelene: 180 ± 47.4; $P > 0.05$). Changes in serum IGF-I concentration are summarised in Table 3.

Discussion

Blunting of the GH response to GHRH in the presence of anticholinergic agents is now well recognised (Casanueva et al. 1986; Pietschmann et al. 1986) and was demonstrated in both groups of subjects. There have been no previous reports of chronic treatment with these drugs on 24-h GH levels.

In a previous study in normal subjects (Taylor et al. 1985), daytime GH secretion was inhibited for 5 h after the acute administration of atropine, 0.6 mg orally, and completely suppressed in two out of four subjects given 1.2 mg by mouth. We gave atropine, 1.2 mg 4-hourly orally, in an effort to abolish GH secretion throughout 24 h. This dose was not effective in totally suppressing 24-h GH levels in normal or diabetic subjects.

With more frequent sampling, particularly at night, we might have been able to demonstrate periods of GH suppression. Nevertheless, the finding of high GH levels in our samples demonstrates that GH secretion was not totally suppressed. This is disappointing, as pituitary ablation was only really effective in the treatment of

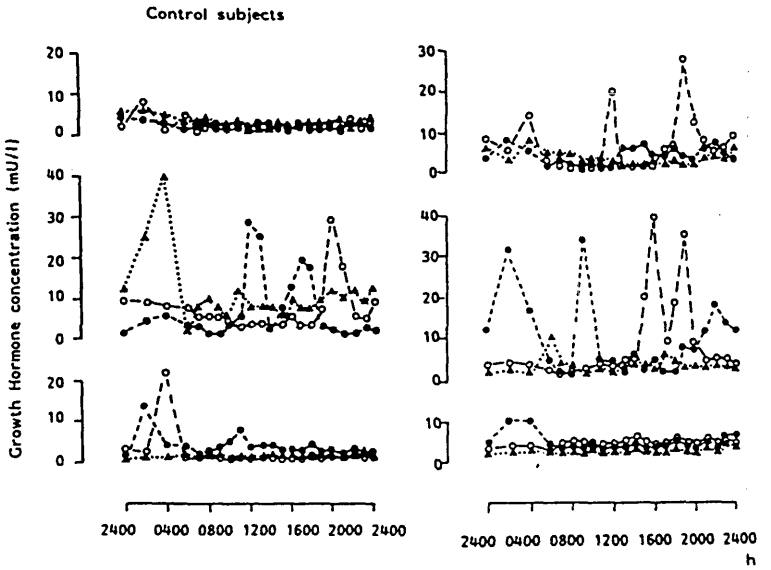


Fig. 2.

24-h GH profiles in 6 normal subjects at baseline (closed circles), after 2 weeks treatment with atropine 1.2 mg × 4 h (triangles) and after 2 weeks treatment with propanthelene 30 mg × 6 h (open circles). Baseline profiles are represented as the mean of 2 control days.

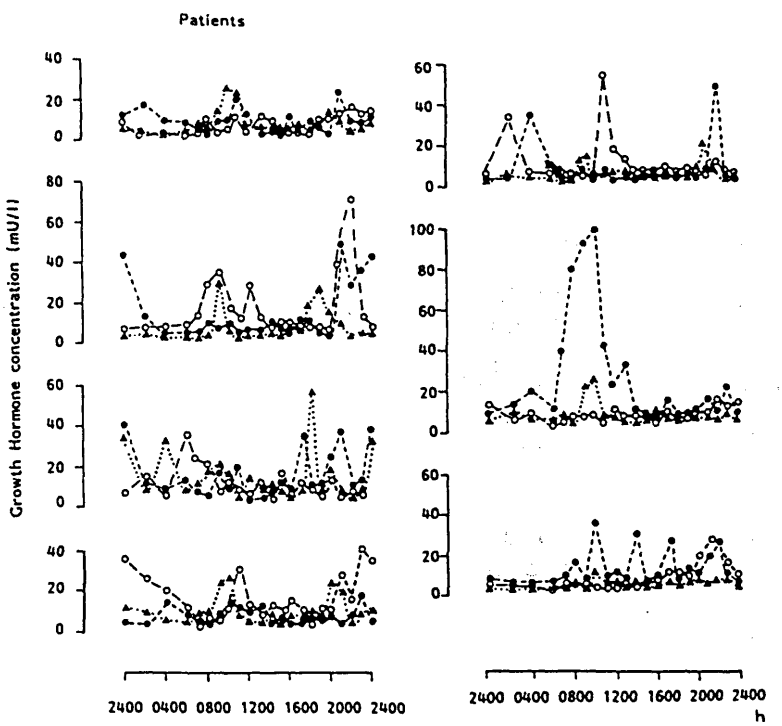


Fig. 3.

24-h GH profiles in 7 diabetic patients at baseline (closed circles), after 2 weeks treatment with atropine 1.2 mg × 4 h (triangles) and after 2 weeks treatment with propanthelene 30 mg × 6 h (open circles).

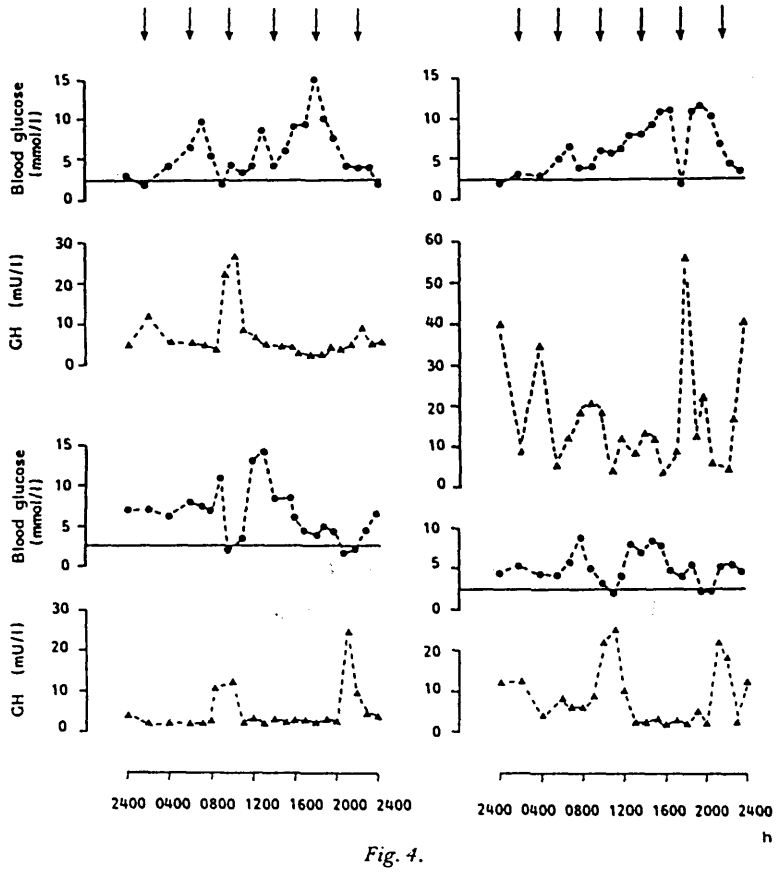


Fig. 4.

24-h GH and plasma glucose profiles in 4 diabetic patients taking atropine 1.2 mg × 4 h as indicated by the arrows. Plasma glucose levels below the line shown are in the hypoglycemic range (< 2.2 mmol/l).

proliferative diabetic retinopathy when GH secretion was totally abolished (Wright et al. 1969; Adams et al. 1974). There was no discernable effect on retinopathy appearance over the study period.

Failure to suppress GH release during hypoglycemia was noted in this study with either treatment and has been previously reported (Blackard & Waddell 1969; Evans et al. 1985). This would make it difficult to maintain total 24-h GH suppression in patients liable to hypoglycemic attacks.

Atropine would not be suitable for long-term use because of severe adverse effects. Propanthelene, a synthetic cholinergic antagonist, was found to be better tolerated than atropine. It has been reported acutely to reduce basal GH levels in normal subjects (Davis et al. 1983). More recently, in a study of four insulin-dependent diabetic men, a significant reduction of night-time GH secretion was observed after a single 45 mg bedtime dose (Davis & Davis 1986).

We did not find propanthelene in the maximally recommended daily dose of 120 mg (British National Formulary 1987) to suppress 24-h GH levels effectively in either the control subjects or

Table 3.

Mean 24-h serum IGF-I μg/l ± SD at baseline, and after atropine and propanthelene.

	Patients	Control subjects
Baseline*	279 ± 136.4	203 ± 47.9
Atropine	195 ± 36.6	166 ± 34.3
Propanthelene	180 ± 47.4	214 ± 57.4

* Mean of two control nights.

in this group of diabetic patients. A lower dose given more frequently might have been more effective.

The acute administration of a similar drug, pirenzepine, abolished sleep-related GH release in five diabetics (Page et al. 1987). In another study, nocturnal GH secretion was inhibited but not totally suppressed, in eight insulin-dependent diabetics with this drug (Martina et al. 1987). The effect of chronic administration of pirenzepine on 24-h GH secretion is not known.

In conclusion, it is unlikely that long-term treatment with atropine or propanthelene will influence the course of diabetic proliferative retinopathy. Whether these agents will be more effective in better controlled patients with milder grades of retinopathy is at present, not known.

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A Two-Year Follow-Up Study of Serum Insulinlike Growth Factor-I in Diabetics With Retinopathy

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Serum insulinlike growth factor-I (IGF-I) concentration was evaluated prospectively over two years in 35 diabetic patients with severe background or preproliferative retinopathy (group 1) and 24 diabetics with mild background retinopathy matched for age, sex, and glycemic control (group 2). In addition, 12 normal subjects were also studied to assess the variability of individual serum IGF-I levels over two years. Mean serum IGF-I (±SD) μg/l at entry, one year, and two years was not significantly different in the patient groups (157 ± 71 *v* 168 ± 77; 166 ± 78 *v* 159 ± 87; 143 ± 58 *v* 159 ± 67) or when compared with the normal subjects (181 ± 47, 188 ± 30; 221 ± 56). Eight patients in the preproliferative group and none in the mild background group developed proliferative retinopathy. In this subgroup developing retinal neovascularization, serum IGF-I at the time of the first appearance of retinal new vessels was significantly higher than 3 months (1 to 4 months) before the onset of proliferation (271 ± 94 *v* 196 ± 58; *P* = .036). Values at the time of proliferation were not, however, significantly different from the mean serum IGF-I value of all patients in group 1 and by 4 months (3 to 6 m) had returned to their previous values. Although a transient elevation of IGF-I occurs at the time of retinal new vessel formation, the rise in serum concentration is not sufficiently great or early enough to be of clinical value as a predictor of retinal neovascularization.

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ELEVATED CIRCULATING and vitreous levels of insulinlike growth factor-I (IGF-I) have been reported in proliferative diabetic retinopathy.[1,2] We and others have not confirmed this finding in serum samples,[3,4] either comparing diabetics with and without retinopathy or diabetics with varying degrees of retinopathy. However, a transient rise in IGF-I concentration occurring just before or at the time of new vessel formation cannot be excluded and might explain the discrepant findings. Such a rise in concentration, if occurring early enough and large enough, might be of clinical value as a predictor of retinal neovascularization as well as having implications for the pathogenesis of proliferative lesions.

We have therefore measured serum IGF-I levels in diabetics with severe background and preproliferative retinal changes (group 1) followed prospectively over two years to assess IGF-I concentration before and after neovascularization has occurred. A group of diabetics with mild background retinopathy (group 2) were studied for comparison.

MATERIALS AND METHODS

Fifty-nine patients attending the Diabetic Retinopathy Clinic at Hammersmith Hospital were studied with a minimum follow-up of two years. All patients had normal serum creatinine (<120 μmol/L). Twelve normal volunteers (six males, six females; 24 to 36 years of age) were also studied to assess the variability of serum IGF-I in normal subjects over two years.

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Retinopathy was assessed by direct and indirect ophthalmoscopy, color photography of both eyes, and fluorescein angiography. At entry to the study, 35 patients had severe background and preproliferative retinopathy (multiple hemorrhages associated with venous beading, cotton wool spots, and intraretinal microvascular abnormalities) equivalent to levels 40/40 to 50/50 on the Wisconsin grading system[5] (group 1). Twenty-four patients with mild background retinopathy (microaneurysms, few hemorrhages, small hard exudates) equivalent to level 30/30 or less were studied for comparison (group 2).

Patients were seen at intervals of 2 to 4 months. Fasting blood samples were taken during the morning clinics for measurement of plasma glucose, hemoglobin A_1 (HbA_1) concentration and serum IGF-I. Urine was also examined for proteinuria by Albustix testing.

Assays

Serum IGF-I was measured by double-antibody radioimmunoassay using the monoclonal antiserum 3D 1/2/1 supplied by Dr R.C. Baxter through the National Hormone and Pituitary Program.[6] The standard was synthetic IGF-I (Amersham, Buckinghamshire), which was labeled with ^{125}I using chloramine-T. Before measurement samples were acid ethanol stripped to separate IGF-I from its binding protein.[7] The limit of detection of this assay was 50 μg/L. At an IGF-I concentration of 200 μg/L, the intraassay and interassay coefficients of variation were 5.3% and 10.2%, respectively. The normal range was 75 to 350 μg/L. Glycosylated HbA_1 was measured by agar gel electrophoresis and is expressed as percentage of total hemoglobin in the glycosylated form (normal range, 3.5% to 5.5%).

Analysis

The two patient groups were compared by an unpaired *t*-test. A stepwise regression analysis was performed to examine the relation between changes in IGF-I and changes in HbA_1 and retinopathy scores in the two patient groups and the subgroup who developed proliferative retinopathy. The relation of IGF-I values and age, duration of diabetes, proteinuria, use of insulin, and body mass index (BMI) was also examined in each group by regression analysis. The predictive value of changes in IGF-I and the onset of proliferation was assessed by discriminant analysis. Significance is taken as $P < .05$. Values are expressed as mean ± SD unless otherwise stated. BMI was calculated as weight (kg)/height (m^2).

Table 1. Clinical Characteristics of Patient Groups

	Severe Background and Preproliferative (Group 1)	Mild Background (Group 2)	P
Age (yr)	47.7 ± 16.4	40.8 ± 13	NS
Sex (M/F)	26/9	20/8	NS
Duration of DM (yr)	16 (2-31)	20 (7-36)	<.02
BMI (weight [kg]/height [cm^2])	26.2 ± 5.0	27.2 ± 4.8	NS
Albustix positive n (%)	21 (60%)	10 (36%)	NS
Insulin-treated n (%)	25 (71%)	24 (85%)	NS
HbA_1 initial (%)	10.55 ± 2.36	10.88 ± 2.07	NS
HbA_1 1 yr (%)	9.5 ± 2.47	10.68 ± 2.11	NS
HbA_1 2 yr (%)	10.38 ± 3.75	9.50 ± 2.12	NS
IGF-I initial (μg/L)	157.1 ± 71	167.8 ± 77	NS
IGF-I 1 yr (μg/L)	165.7 ± 78.4	158.9 ± 87.2	NS
IGF-I 2 yr (μg/L)	142.8 ± 58	158.9 ± 67	NS

Abbreviation: DM, diabetes mellitus.

RESULTS

The two patient groups were similar in respect to sex distribution, age, BMI, prevalence of proteinuria, and use of insulin (Table 1). The patients in group 1 had significantly shorter duration of diabetes when compared with those in group 2 [median (95% confidence limits), 15 years (range, 12 to 17) *v* 20 years (range, 16 to 23); $P = .02$].

The mean ± SD of the individual coefficients of variation of serum IGF-I over the two-year period was 9.6% ± 6.2% in 12 normal volunteers, 22.6% ± 15.7% in the mild retinopathy group, and 25.8% ± 17.3% in the severe background and preproliferative group ($P < .05$ controls *v* patients). At entry, one year, and two years, mean serum IGF-I was 181 ± 47, 188 ± 30, and 221 ± 56 μg/L, respectively, in the normal volunteers.

Mean IGF-I concentration (μg/L) at entry, one year and two years was similar in both groups of patients (157 ± 71 *v* 168 ± 77 at entry; 166 ± 78 *v* 160 ± 87 at one year; and 143 ± 58 *v* 160 ± 67 at two years; NS). These values were also not significantly different when compared with the mean yearly IGF concentrations in the normal subjects. There was no correlation between IGF-I values and severity of retinopathy within either patient group. Mean HbA_1 values were similar in both groups of patients at entry, one year, and two years (Table 1).

During the study period, eight patients in group 1 and none in group 2 developed proliferative changes; new vessels occurred in all quadrants in six patients and in only one quadrant in two patients. Clinical details of these patients are shown in Table 2.

Changes in serum IGF-I in the eight patients developing proliferative retinopathy are shown in Fig 1. Values before proliferation (median, 3 months (range, 1 to 4 months) are at the time of the last visit before the recognition of proliferative lesions. Mean IGF-I concentration at the first appearance of new vessels was significantly higher compared with the value at the previous visit (271 ± 94 *v* 196 ± 58 μg/L; $P = 0.36$). The mean value at the onset of proliferation was not, however, significantly different when compared with the mean for all group 1 patients at entry, one year, or two years.

Table 2. Clinical Details of Patients Developing Proliferative Retinopathy

Patient	Sex	Age (yr)	Duration of DM (yr)	BMI	Type	Proteinuria (g/L)
1	F	24	22	22.3	IDDM	2
2	F	27	17	22.9	IDDM	1
3	M	29	21	23.6	IDDM	3
4	M	36	9	34.7	IDDM	3
5	M	33	18	23.0	IDDM	1
6	F	24	20	20.9	IDDM	2
7	M	68	6	23.7	NIDDM	0
8	M	54	5	28.2	NIDDM	0

Abbreviations: IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; DM, diabetes mellitus.

Patients 1 through 6 developed new vessels in all quadrants; patients 7 and 8 in only one quadrant.

The onset of proliferation could not be predicted by changes in IGF-I level. The combination of a rise in IGF-I and a fall in HbA_1 correctly identified six of the eight patients who developed proliferation but falsely classified ten patients in group 1 and four in group 2 into the proliferative group.

No significant correlation was observed between serum IGF-I values and age, sex, BMI, HbA_1 concentration, duration of diabetes, insulin treatment, or prevalence of proteinuria in either group of patients. Changes in HbA_1 concentration in the subgroup developing proliferative retinopathy are shown in Table 3; there was no significant correlation between changes in IGF-I values before and after proliferation and changes in HbA_1 level ($P > .05$).

DISCUSSION

In this study, a significant elevation in IGF-I concentration was observed at the time of proliferation, especially in

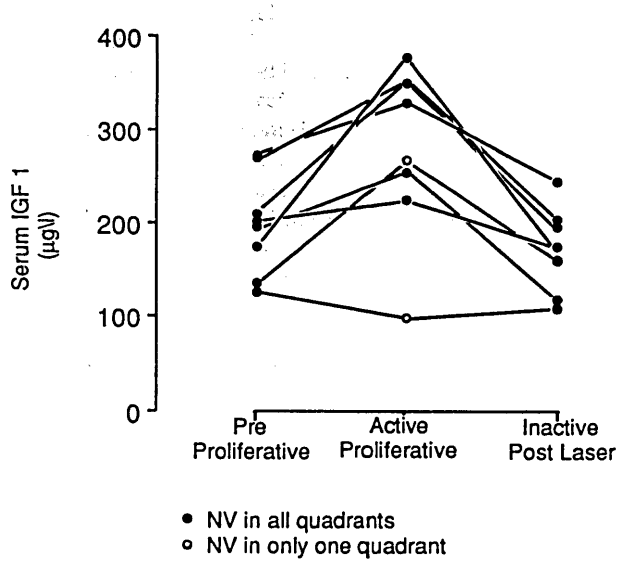


Fig 1. Changes in serum IGF-I concentration in eight patients developing proliferative retinopathy. The figure shows values at the last visit (3 months [1 to 4 months]) before proliferation was apparent, values at the first appearance of retinal new vessels, and values after successful laser treatment (4 months [2 to 6 months]).

Table 3. Changes in Serum IGF-I Values (μg/L) and HbA_1 Concentration (%) in Patients Progressing From Preproliferative to Proliferative Retinopathy

Retinopathy Patient No.		Preproliferative	Active Proliferative	Inactive Postlaser
1	IGF-I	175	376	175
	HbA_1	12.9	8.3	9.2
2	IGF-I	270	350	196
	HbA_1	16.0	5.2	6.6
3	IGF-I	271	329	245
	HbA_1	15.0	12.1	15.1
4	IGF-I	196	255	119
	HbA_1	13.4	15.3	17.0
5	IGF-I	202	225	174
	HbA_1	13.7	6.0	9.3
6	IGF-I	210	350	203
	HbA_1	9.3	7.7	9.7
7	IGF-I	126	99	109
	HbA_1	7.6	7.9	3.7
8	IGF-I	135	264	168
	HbA_1	9.8	9.2	9.6

younger patients with new vessels in all quadrants, but only one patient had values outside the normal range. This is in contrast to patients with rapidly accelerating retinopathy who have been reported to have very high serum IGF-I values.[1] The same group[2] found a correlation between serum and vitreous IGF-I levels in diabetic patients with proliferative retinopathy but not in nondiabetic control subjects, suggesting that IGF-I enters the vitreous in patients by leakage from blood vessels.

Because all the patients developing proliferative retinopathy showed extensive leakage of fluorescein at angiography and vitreous fluorophotometry, it is possible that vitreous levels were even higher than serum levels would suggest. However, it cannot be assumed that leakage on angiography or fluorophotometry correlates with leakage of IGF-I because these techniques show only leakage of free fluorescein (molecular weight, 376) and IGF-I circulates largely bound to carrier proteins.[8]

IGF-I values tended to be more variable in the diabetic group presumably reflecting fluctuations in diabetic control.[9] Acid-ethanol extraction may not have completely removed all binding protein,[10] and this may also have contributed to the variability of IGF-I measurements. Individual IGF-I levels or a combination of improved control (fall in HbA_1) and increase in IGF-I were unable to predict deterioriation in retinopathy.

With less variability of IGF-I levels, values at the onset of proliferation may have proven significantly greater than the mean for all the patients with preproliferative retinopathy. However, the rise was not early enough to be of clinical value as a predictor of progression of retinopathy.

Although the rise in IGF-I concentration at the time of proliferation did not appear to relate to changes in glycemic control, six of the seven patients in whom IGF-I levels increased showed a simultaneous reduction in HbA_1 levels, indicating improved diabetic control. The rise in serum IGF-I may be consequent upon an improved use of metabolic substrates analagous to the increase in circulating levels occurring after refeeding malnourished children.[11] A similar rise in serum IGF-I occurs shortly after commencing continuous subcutaneous insulin infusion (CSII).[12]

In two patients (patients 2 and 5) a dramatic improvement in diabetic control preceded the development of neovascularization. Rapid worsening of retinopathy associated with the sudden institution of tight glycemic control has been reported during CSII[13-15] and after pancreas transplantation.[16]

The mechanism responsible for progression to proliferative retinopathy is not known. Retinal ischemia might result from a reduction in hyperglycemia-enhanced retinal blood flow.[17] IGF-I may then act synergistically with retinal-derived growth factors[18,19] produced by the ischemic retina to enhance new vessel formation.

There is good evidence that IGF-I binds to specific receptors on retinal endothelial cells and stimulates DNA synthesis.[20] IGF-I has also been shown to be chemoattractant to endothelial cells[21] so that increased local IGF-I concentrations in the vitreous, for example, might attract new vessel growth.

Recently, insulin has been shown to regulate serum levels of the 34-kd IGF-I binding protein, levels of this protein being elevated in diabetes and decreased by insulin treatment.[22] The physiologic significance of this finding at the level of the retinal endothelial cell is not known but could provide a mechanism for increased IGF-I activity during improved diabetic control.

In summary, we have not found serial measurements of IGF-I at intervals of 2 to 4 months to be of clinical value in predicting patients likely to develop proliferative retinopathy. However, the transient rise in serum concentration about the time when new vessels were first observed is additional evidence for a role of IGF-I in the development of diabetic retinal neovascularization.

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Human diabetic serum does not stimulate bovine retinal endothelial cell growth in culture

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ABSTRACT

Bovine retinal microvascular endothelial cells were cultured with serum from nondiabetic controls, patients with background, preproliferative, proliferative and inactive (treated) proliferative diabetic retinopathy. Using Coomassie Blue to measure cellular protein, we found no significant increase in retinal endothelial cell growth with serum from patients with preproliferative or proliferative retinopathy.

INTRODUCTION

Proliferative diabetic retinopathy continues to be a major cause of blindness (1,2). Whilst it is thought that the new blood vessels involved are stimulated by growth factors produced in the surrounding ischaemic retina, the role of serum factors is unknown (3,4,5). Diabetic serum has been shown to stimulate endothelial cells (6) and studies on human umbilical vein endothelial cells cultured in the presence of serum from patients with proliferative retinopathy demonstrated increased growth (7). Koschinsky demonstrated increased growth of human skin fibroblasts and smooth muscle cells in serum from poorly controlled non-insulin dependent diabetics, whilst Hauss and Ledet showed increased rat and rabbit aortic smooth muscle cell growth (8,9,10,11).

This study seeks to determine if serum from diabetic patients with proliferative retinopathy also stimulates retinal endothelial cell growth, which would be of direct relevance in the evolution of diabetic retinopathy.

MATERIALS AND METHODS

Serum samples

Blood was collected from 35 patients attending the Diabetic Retinopathy clinic and 8 non-diabetic control subjects, one healthy subject provided a reference sample against which all samples were compared. All sera were aliquoted and stored at -20°C, thawed just before use, and used at a concentration of 10% in culture medium.

The sera of 27 patients and 8 controls were examined against the reference serum. Of the patients, 6 had background retinopathy, 8 preproliferative, 6 active proliferative and 7 treated, inactive, proliferative retinopathy. A further 4 sera from patients with active proliferative retinopathy were compared against 4 with background retinopathy.

Microvessel isolation

Bovine retinal microvessels were isolated weekly throughout the study, and only cells from the first subculture were used. The culture techniques were similar to those developed by McIntosh et al (12), and Wong et al (13). Seven bovine eyes were rinsed in 20% betadine, and the retinae dissected within 24 hours of the animal's death. After rinsing in Minimum Essential Medium (MEM, GIBCO, UK: 041 02360) at 4°C, the retinae were homogenised with a Teflon/glass homogeniser (Jencons, UK) to produce a microvessel suspension. This was then filtered onto three 85 μm filters (Henry Simon Ltd, Stockport, UK). The vessels on the filter were removed, suspended in a solution of collagenase-dispase (Boehringer Mannheim, FRG: 269638), 1 mg/retina in MEM, for 1 hour at 37°C and shaken to prevent clumping. After digestion, Hanks Buffered Salt solution (HBS) 10% HBS X 10 without calcium or magnesium, 5 mmol/l Hepes, 27 mmol/l bicarbonate, 100 iu/ml

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penicillin and 100 µg/ml streptomycin), at 37°C, was added to dilute the enzyme before centrifugation at 1400 rpm for 6 minutes. All the medium components were obtained through GIBCO, UK, unless otherwise stated, and plastics from Falcon, UK.

Cell culture

After centrifugation the pellet was re-suspended in 15 ml medium containing 10% plasma derived serum, 8% Glasgow's Modified Eagles Medium X10, 10% tryptose-phosphate broth, 20 mmol/l bicarbonate, 45 mg/100 ml glutamine, 1 mmol/l Hepes, 0.55 mmol/l NaOH, 100 iu/ml penicillin and 100 µg/ml streptomycin to pH 7.3-7.4, known as 10% PDS. The cells were plated onto fibronectin coated flasks, gassed in 95% air/5% CO2, and sealed. The next day the cells were washed (HBS), and fresh medium added (8% PDS: prepared as 10% but with 8% plasma-derived serum).

Human fibronectin was obtained from New York Blood Centre, made up to 50 µg/ml with phosphate-buffered saline (PBS: Oxoid, without calcium and magnesium), and plated at 37°C using 3 ml/75 cm2 flask, three hours before the cells were added. The PDS was prepared as that of McIntosh: pooled frozen serum was obtained from a local blood bank, dialysed at 4°C against two changes of buffered salt solution, filtered (0.2 µm), and stored frozen.

Trypsinisation procedure

After 5 days the primary culture of endothelial cells had almost attained confluence, and were then trypsinised. The medium was removed, and the cells washed twice with HBS. To detach the cells 6 ml trypsin (2.5% Flow, Cat no: 16-893-49: diluted further with HBS to 0.25%, 20°C) was added with mild agitation for approximately 1 minute. 20% calf serum medium, prepared similarly to the 10% PDS medium, was added to neutralise the trypsin, the cells gently pipetted for 2 minutes and then centrifuged at 1250 rpm for 6 minutes. They were then re-suspended in 2% heat inactivated foetal calf serum (2% HI) again prepared similarly to the 10% PDS medium, and gently syringed through a 21 gauge needle, centrifuged again (1250 rpm for 6 minutes) and re-suspended in 2% HI serum. After mixing with 0.2% trypan blue, the viable cells were counted, using a Neubauer chamber; 1-2 X 106 cells per flask were obtained.

96 well plates

Fibronectin was added to each well (50 µl/well) and three hours later 100 µl test medium, followed by 50 µl cell suspension, the final serum concentration was 5%. Using sterile water in the outer rim to prevent evaporation, two serum samples were tested on each plate using 20 wells for each, with 20 wells for the reference serum.

Several problems had to be overcome at the trypsinisation stage: confluent cultures produced clumped cells when trypsinised, and temperature gradients between the plate and the cell suspension, caused uneven cell distribution within wells. Such plates were discarded.

Fibroblast growth factor

Recombinant basic fibroblast growth factor (bFGF), (Amersham, Bucks, UK), was reconstituted in sterile distilled water and made up with 10 mmol/l Tris: HCl (pH 7.4) to 50 ng/ml: 30 µl of this was diluted with 5 ml 10% PDS medium and added to the wells to give a final concentration of 150 pg/ml.

Coomassie blue stain

After 2 days the subcultured cells were 2/3 confluent and were stained: wells were rinsed with PBS 4 times, each time exchanging 100 µl (half their volume), to avoid washing cells off. The PBS was removed, 100 µl Coomassie Blue G250, 0.04% in 3.5% perchloric acid (12,14), added. After 5 minutes this was replaced with a further 100 µl Coomassie Blue. This was then left overnight before measuring the increase in absorbance (620 nm) compared to a well containing Coomassie Blue alone, using a Titertek Multiscan plate reader (Flow, UK).

Tritiated thymidine incorporation

Cells from the same trypsinisation were plated onto 96 well plates and grown in 5% and 2% plasma-derived serum (the 2% medium was prepared as the 5%, but diluted 3:2 with serum free medium). After 24 hours 1 μCi of 3H-Thymidine (Amersham) was added (diluted to 20 μl with 2% medium). The Thymidine incorporation was measured after a further 24 hours using the method described by Morgan (15): after PBS washes (as above), cells were washed with 5% trichloracetic acid. After 5 minutes this was removed and after a wash with methanol the cells were digested with 25 M formic acid and the formic acid digest placed into 1 ml of scintillation (Optiphase, LKB, UK). Each well was further washed with PBS, and this wash was added to the respective scintillant vials and the radioactivity measured (LS 1800, liquid scintillation counter, Beckman, USA).

Characterisation

Cells from the primary subculture were grown in 8% PDS medium on Lab-Tek tissue culture slides (Flow, UK) and were immunostained for von-Willebrand factor (Factor VIII-related-antigen) using rabbit anti-human von-Willebrand antiserum and fluorescein-conjugated goat anti-rabbit Immunoglobulin. Negative controls used non-immune goat serum (all reagents from Dako, UK). Bovine retinal pericytes were also stained for comparison.

Statistical method

All results were compared using unpaired t-tests.

RESULTS

Staining for von-Willebrand factor

The endothelial cells were differentiated from pericytes by their typical cobblestone morphology and their uniform staining for von-Willebrand factor, for which the pericytes did not stain.

Proliferative assays

A linear increase in Coomassie Blue staining between 24 and 72 hours after seeding was observed (fig 1), with confluence reached after 60 hours. The increase was similar in diabetic and pooled normal serum. Staining was directly proportional to the original cell number (fig 2) whether cells were stained at 3 or 48 hours, however, as expected cell growth was more rapid at a higher seeding concentration. To test response to growth factors, cells from the same trypsinisation procedure used for the serum experiment were treated by the addition of 150 pg/ml bFGF. They showed a

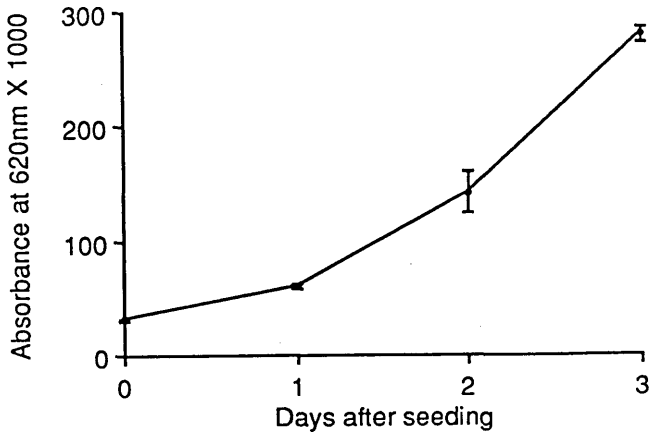


Figure 1: Daily increase in absorbance after Coomassie Blue staining. Cells seeded at 4000/well. The spectrophotometer was blanked on wells containing Coomassie Blue alone. Bars show standard error of the mean.

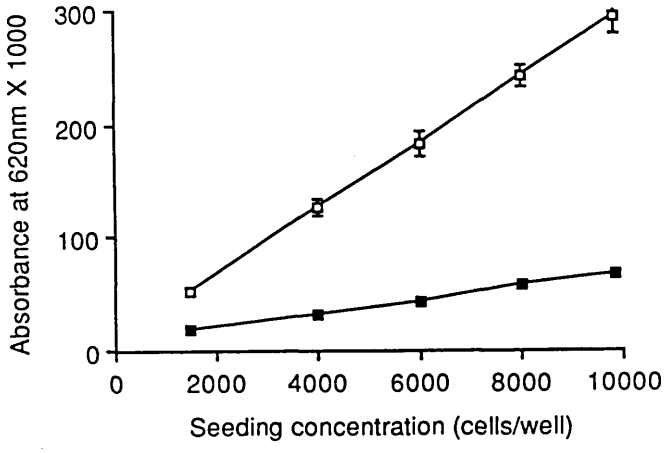


Figure 2: Increase in absorbance after Coomassie Blue staining with increased seeding concentration: Cells were stained at 3 (closed squares) and 48 hours (open squares) after seeding. Bars show standard error of the mean.

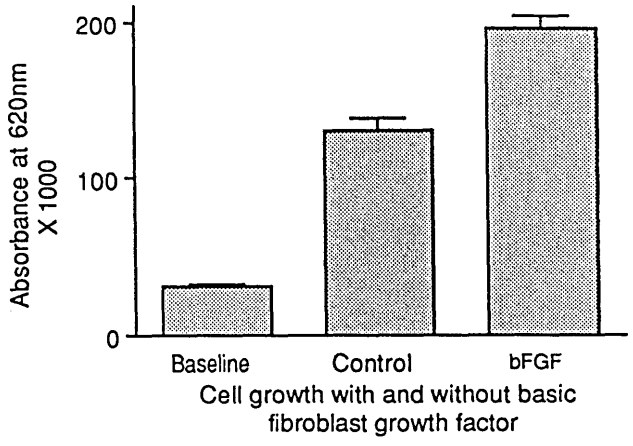


Figure 3: Increased absorbance after Coomassie Blue staining with the addition of basic fibroblast growth factor (bFGF). Cells were seeded at 4000/well and stained after 3 hours (baseline) or 48 hours. Bars show standard error of the mean.

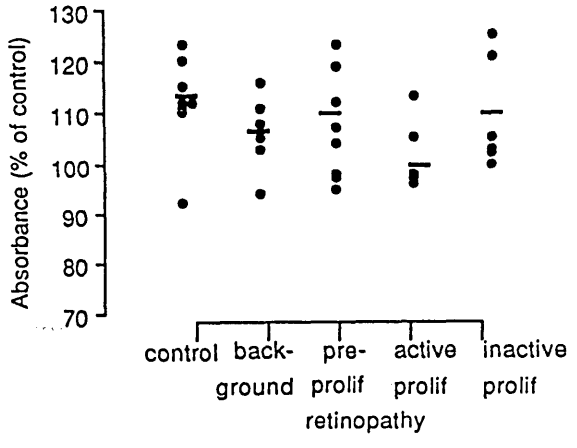


Figure 4: Increased absorbance after Coomassie Blue staining: serum samples were compared to a reference serum, and the result expressed as a percentage. (4000 cells/well, stained after 48 hours). Each dot represents the mean absorbance (mean of 20 wells) from one patient or control serum. The bars show the mean value of each group. The X axis refers to the type of retinopathy the patient had when the serum sample was taken (Prol = proliferative).

50% increase in absorbance after Coomassie Blue staining (fig 3).

Comparing Thymidine incorporation with Coomassie Blue staining, cells were grown in 2% and 5% PDS medium.

Thymidine incorporation between 24 and 48 hours expressed as cpm ± SD (n=20) was 26,500 ± 3,800 at 2% PDS and 31,300 ± 2,900 at 5% PDS an increase of 18% ($P < 0.05$). The increase in Coomassie Blue staining between 5 and 2% PDS was 29% ($P < 0.05$).

Cell growth in diabetic serum

The mean absorbance value of the 20 wells for each serum sample or control serum was calculated and showed a standard deviation of between 6% and 14%. 2% of wells had many cells washed off and were excluded. The result for each patient's serum was calculated by comparing the mean absorbance with that of the reference serum on the plate and expressing it as a percentage of the reference. These results show no significant difference between the serum samples from the four patient (fig 4) and the control groups ($P > 0.05$). Additionally, a paired study was performed to directly compare sera from patients with proliferative and background retinopathy: there was no significant difference in absorbance between the two groups (four patients in each group) ($P > 0.05$).

DISCUSSION

Several factors in serum could stimulate endothelial cell proliferation. King et al (16) demonstrated that Insulin-like growth factor (IGF-I) stimulated bovine retinal endothelial cells in a dose dependent manner. In a prospective study (17), Hyer et al showed that circulating levels of IGF-I were increased at the time of new vessel formation.

In a recent study (7), serum from patients with neovascularisation stimulated human umbilical vein and omental endothelial cell growth significantly more than serum from patients with background retinopathy. However, serum stimulatory activity did not correlate with IGF-I levels.

In this study, we found no increase in the growth of bovine retinal endothelial cells exposed to sera from patients suffering from background, pre-proliferative, proliferative or inactive (treated) proliferative

retinopathy. Methodology differences between these and other studies (6,7) are unlikely to be responsible for differences between our results. Culture techniques were based on those of McIntosh et al (12) and the Coomassie blue staining procedure was found to correlate with results obtained by 3H-Thymidine incorporation. The fact that 3H-Thymidine incorporation was carried out over 24 hours and the Coomassie Blue staining at 48 hours may make comparison between the two difficult. However, a 29% increase in cell growth between 2 and 5% PDS observed with Coomassie blue against an 18% increase in 3H-Thymidine incorporation suggests that the former technique is at least as sensitive as 3H-Thymidine incorporation in measuring cell growth response. Also the successful measurement of bFGF-stimulated cell growth shows that the technique is viable.

It is possible that differences in cell types used in this and other studies (8-11) could account for the different results. It cannot be assumed that smooth muscle cells will respond similarly to bovine retinal endothelial cells.

Furthermore, endothelial cells from retinal microvessels differ morphologically and metabolically from those found in other tissues (13,18). However, species differences are likely to be more important. Human endothelial cells as opposed to bovine endothelial cells were used in other studies (6,7). In support of this, 5 of our sera from patients with proliferative retinopathy showed a 205% ± 27.1% (SEM) increase in 3H-Thymidine incorporation when compared to control sera using human umbilical endothelial cells. In contrast bovine aortic endothelial cells showed no increase in ^{3}H-Thymidine incorporation when compared to control sera (Dr R Petty - personal communications).

The fact that bFGF stimulates BREC growth but that diabetic sera, whilst stimulating ^{3}H-Thymidine incorporation into human umbilical endothelial cells, does not promote BREC growth, indicates that the stimulatory factor in diabetic sera is unlikely to be bFGF.

In summary, whilst serum from patients with proliferative retinopathy stimulates many cell types, it has no major effect on the growth of bovine retinal endothelial cells. That species differences account for our findings is likely. How human retinal endothelial cells in culture will respond remains to be seen.

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