

VEIN GRAFT STENOSIS AND THE HUMAN VASCULAR SMOOTH
MUSCLE CELL

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ABSTRACT

Vein graft stenosis is one of the most important barriers to long term patency following bypass grafting. Its incidence has been variously reported between 20 and 30%. The failures of vascular intervention are caused by excessive myointimal hyperplasia (MIH) in 70 - 80% of cases and human vascular smooth muscle cell (VSMC) proliferation is a key part of this process. Human VSMC cultured from an individual display consistent proliferative responses to heparin but VSMC from different individuals display diverse responses.

I have used a human cell culture system to further investigate the biology of MIH and shown, prospectively, that vascular smooth muscle cells cultured from patients with stenotic grafts are significantly less sensitive *in vitro* to growth inhibition by heparin than those from patients with patent grafts and also have fewer binding sites for tritiated- heparin.

I have also looked at the interaction between peptide growth factors (particularly PDGF) and human VSMC and demonstrated that they play a highly prominent role in the co-ordination of VSMC replication and migration and this occurs via a subtle interplay of proliferative and chemotactic signals.

In conclusion the reduced responsiveness of the saphenous vein derived human VSMC to heparin appears to be one of the strongest markers yet for vein graft stenosis.

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For Sarah and Joshua

"Out of clutter, find simplicity
out of discord, find harmony
in the middle of difficulty, lies opportunity"
Albert Einstein

Publications arising from this work to date:

PAPERS

1] CHARACTERISATION OF [³H]- HEPARIN BINDING IN HUMAN VASCULAR SMOOTH MUSCLE CELLS AND ITS RELATIONSHIP TO THE ANTIPROLIFERATIVE EFFECT.

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Gen Pharmacol 1996 Oct;27(7):1079-1089.

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1] CORRELATION OF HEPARIN BINDING WITH RESPONSIVENESS IN HUMAN VASCULAR SMOOTH MUSCLE CELLS.

J Refson, M Schachter, M Patel, P Chan, JHN Wolfe, P Sever.

Biochem Soc Trans 1995 (23) 172S.

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Inter. Angio 1995; 14 (Suppl. 1): 124.

5] DIFFERENTIAL MITOGENIC EFFECTS OF PDGF ISOFORMS AND HEPARIN ON HUMAN VASCULAR SMOOTH MUSCLE CELLS.

J Refson, M Schachter, M Patel, JHN Wolfe, P Sever.
Inter. Angio 1995; 14 (Suppl. 1): 141.

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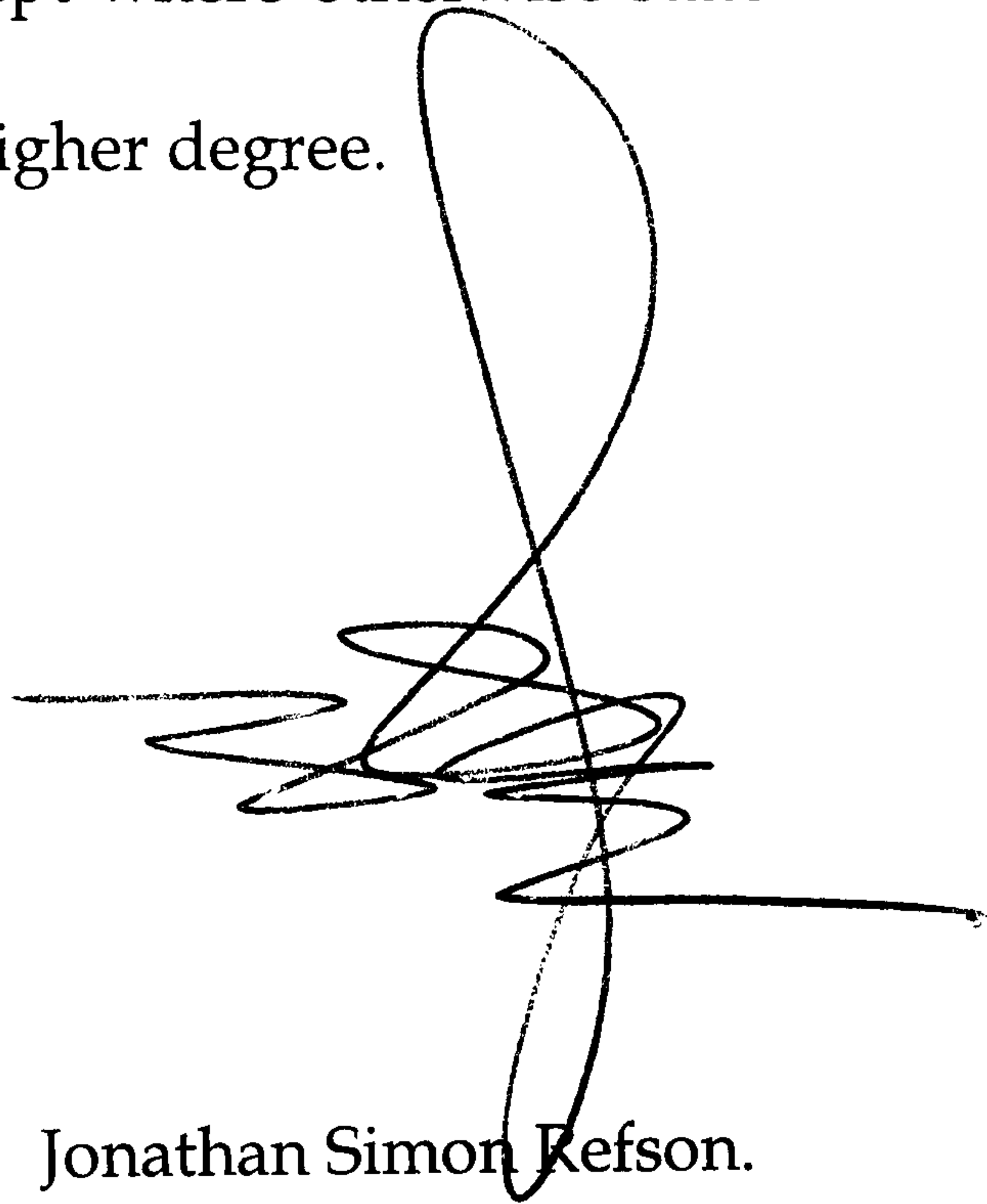
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DECLARATION

I hereby declare that I am the author of this thesis and that all references cited have been consulted by me. Furthermore, all the work reported in this thesis was performed by me except where otherwise stated. This work has not been previously accepted for a higher degree.

A handwritten signature in black ink, consisting of a large loop at the top, followed by several horizontal and vertical strokes, and ending with a long horizontal line extending to the right.

Jonathan Simon Refson.

PERIPHERAL VASCULAR DISEASE.

ATHEROSCLEROSIS:

Atherosclerosis is defined as a disease of large and medium sized arteries, in which the intima of the arterial wall is thickened by the development of fibrous tissue and accumulation of lipid. (Greek, athere- gruel or porridge; sclerosis-hardening). The disease has a predilection for the elastic arteries particularly the aorta and its branches. The clinical manifestations of the disease result from the narrowing of these arteries. The atheromatous plaque may ulcerate and thrombose, embolism or vessel occlusion may ensue, with consequent acute or chronic ischaemia of the end organ supplied. Disease affecting the arteries to the brain, heart and legs is a major clinical problem, with the ultimate results being stroke, myocardial infarction and limb loss respectively (Taussig 1984).

In fact, the magnitude of this clinical problem cannot be emphasised strongly enough, since it is responsible for 50% of all mortality in Europe, USA, and Japan (Ross 1993) and 156,000 deaths in England and Wales each year are due to ischaemic heart disease, accounting for 30% of all male deaths and 22% of all female deaths (Watt and Spence 1986).

THE LESIONS OF ATHEROSCLEROSIS:

The earliest recognisable lesion in atherosclerosis is the “fatty streak”, which is an aggregation of lipid rich macrophages and T lymphocytes within the innermost layer of the artery wall, the intima. This lesion has been seen in newborn Watanabe heritable hyperlipidaemic rabbits, which suggests the atherosclerotic process may commence *in utero* (Aliev et al. 1993). In man these lesions have been found in post-mortem specimens from children as young as 10 years old (Stary 1989). The fatty streak precedes the development of intermediate lesions (Fagiotto et al. 1984) which are composed of layers of smooth muscle cells and macrophages and in-turn these develop into the mature complex lesions that are called fibrous plaques. These also consist of smooth muscle cells and lipid laden macrophages but in addition there is collagen and elastin fibers and abundant extracellular matrix, which forms a fibrous cap over the lesion. Most sudden deaths from myocardial infarction are due to rupture or fissuring of the plaque margin, where there are relatively more macrophages, this results in intraplaque haemorrhage and subsequent thrombosis and occlusion of the artery (Davies et al. 1984). Plaque rupture or disruption and thrombosis is therefore, primarily responsible for lethal acute coronary events.

Plaques with a large extracellular lipid-rich core, thin fibrous cap with reduced collagen content and smooth muscle density, and increased numbers of activated macrophages and mast cells appear to be most vulnerable to rupture. Plaque disruption tends to occur at points at which the plaque surface is

weakest and most vulnerable, which coincide with points at which stresses, resulting from biomechanical and haemodynamic forces acting on plaques, are concentrated (Shah 1996).

PATHOGENESIS:

The key cellular events that occur in the development of a plaque are: (i) proliferation of smooth muscle cells, macrophages and possibly lymphocytes. (ii) formation by smooth muscle cells of extracellular matrix and (iii) accumulation of free and esterified cholesterol and lipid in the matrix and surrounding cells. There have been many theories regarding the development of atherosclerotic lesions, the most widely accepted being the response to injury (to the endothelium) hypothesis. This was first proposed by Virchow in 1856 and has subsequently been refined with the advent of new observations originally put forward by Ross et al. (1973) and subsequently advanced upon by him more recently in 1993 (Ross 1993). This theory proposes that quiescent smooth muscle cells in the vessel wall are stimulated to replicate following exposure to platelet derived factors following damage to the vessel endothelium. The proliferative responses and cellular aspects of the complex fibrous lesions are a variation on the normal healing process. The response of the arterial wall differs primarily in that the principal source of connective tissue in the artery wall is the vascular smooth muscle cell rather than the fibroblast (as in other tissues) and secondly that the injurious agents (smoking, diabetes, hypercholesterolaemia and hypertension) are ongoing and therefore,

likely to behave in a chronic manner, allowing uninterrupted progression from fatty streak to complicated fibrous plaque. The location of these lesions primarily at branching points in the arterial tree may be as a result of haemodynamic stresses and the presence of one or more of the above risk factors allows progression of the lesion (Glagov et al. 1989).

Many cytokines and growth factors such as Platelet derived growth factor (PDGF) (Walker et al. 1986), basic Fibroblast growth factor (bFGF) (Folkman et al. 1988), Insulin like growth factor-1 (IGF-1), (Banskota et al. 1989), epidermal growth factor (EGF) (Miyagawa et al. 1995) and interleukin-1 (IL-1) (Raines et al. 1991) to name but a few that are not normally expressed in normal artery have been found to have upregulated expression in atherosclerotic lesions. These factors can all induce cellular proliferation and migration, and synthesis of lipids, proteins and extracellular matrix. Their action is probably exerted through a network of cellular interactions, the action of one molecule leading to the expression of a second in a target cell that can either stimulate neighbouring cells in a paracrine way or in an autocrine fashion on itself. The role of the growth factors will be discussed further when dealing with restenosis.

EPIDEMIOLOGY OF INTERMITTENT CLAUDICATION

Atherosclerotic changes in the vessel wall begin to take place in second and third decades of life if not even earlier. Dilatation of collateral blood vessels represents a physical adaptation to the stenotic process induced by

atherosclerosis. But, as blood flow is dependent on the radius of a vessel raised to the fourth power, collateral circulation cannot fully compensate for the diminished muscle blood supply during exercise. The insufficient circulation causes muscle ischaemia and pain. Intermittent claudication is usually the first symptom of peripheral vascular disease. It is typified by a cramp like pain in the calf brought on by walking and relieved by resting. The most usual location of the causative lesion in these individuals is in the superficial femoral artery. The claudication may also occur in the thigh and buttock and be associated with impotence as a result of iliac artery disease. This is known as Leriche syndrome (Leriche and Morel 1948).

INCIDENCE

From the Edinburgh Artery Study the prevalence of IC was found to be 4.5% with no significant difference between the sexes (Fowkes et al. 1991). From the Framingham study the incidence of intermittent claudication varies from 3.6/1000 for women to 7.1/1000 for men per two years, with the relative risk of a patient with claudication developing ischaemic heart disease at 2.3 for men and 5.4 for women (Kannel and McGee 1970). In a follow up study on this same population comparing claudicants with non-claudicants it was demonstrated that claudication is an expression of generalised atheromatous disease, with claudicant mortality being double that of non claudicants (Kannel and McGee 1985). In a more recent 7 year follow up study of claudicants, 33% were dead at

the end of the follow-up period, 44% due to myocardial infarction and 28% due to cerebrovascular disease (O'Riordain and O'Donnell 1991).

RISK FACTORS

A number of risk factors for claudication have been identified from case control and cohort studies. The two most obvious from the above data are male gender and increasing age. The following also have all been shown to be causative independently and in combination: smoking, hypertension, hypercholesterolaemia, and diabetes. These risk factors have also been evaluated for their involvement in the generation of the myointimal hyperplasia (MIH) (See chapter 2).

SMOKING

This is one of the predominant factors in the development of cardiovascular disease. The risk of sudden death due to coronary heart disease in smokers is twice that of non smokers. In a case control study the incidence of intermittent claudication is nine times higher in individuals who smoke more than 15 cigarettes per day than in non smokers and six times higher in those smoking from one to fourteen cigarettes per day. Pipe and cigar smokers also had a seven fold increase in risk of intermittent claudication compared to non smokers.(Hughson et al. 1978). In a five year follow up of smoking claudicants

and non smoking claudicants, those that gave up had an amputation rate of zero and those that continued had one of 11.5%. This tendency is borne out in relation to possibility of stroke due to carotid artery disease (Abbott et al. 1986) and myocardial infarction due to coronary atherosclerosis. From multivariate analysis from the Framingham study, smoking is the most important single risk factor for the development of IC.

DIABETES MELLITUS

From the Framingham study, peripheral occlusive disease is twice as common in diabetic men and three times more common in diabetic women than in controls and diabetic men are nearly four times more likely to develop IC than non-diabetics (Kannel and McGee 1979). The presence of critical ischaemia is also five times more likely in diabetics (Second European Consensus Document on Chronic Critical Limb Ischaemia 1991). This makes diabetes the second most important single risk factor for IC.

HYPERTENSION

A study examining peripheral arterial disease in the sixty plus age group revealed prevalence of 26.7% among hypertensives. Again, analysis of data from the Framingham study reveals that hypertension (two standard deviations from the mean) is much more strongly correlated with stroke (four times), and

myocardial infarction (twice), than IC (only one and a half times) (Gordon and Kannel 1972). However, systolic pressure is more closely correlated than diastolic for IC with a three fold increase in risk.

CHOLESTEROL AND LIPIDS.

The evidence for the involvement of an elevated cholesterol in the development of coronary heart disease is beyond question (Slack 1969), but, its role as a risk factor in the development of IC is far from clear. Hughson et al. (1978) and Greenhalgh et al. (1971) were unable to determine a significant difference in cholesterol concentrations between claudicants and controls. However, data from the Framingham cohort revealed an increased incidence of high cholesterol among claudicants (Gordon and Kannel 1972). Interestingly, elevated triglycerides were also more common among claudicants than controls in the Slack study (1969), although, this was not controlled for diabetes.

FIBRINOGEN AND URATE

Higher levels of fibrinogen have been found in patients with peripheral vascular disease (Hughson et al. 1978 and Dormandy et al. 1973). However, this may reflect the smoking habits of patients rather than represent a true independent risk factor (Fowkes 1988).

Hughson et al.(1978) and Dormandy et al. (1978) also reported raised urate levels in patients with peripheral vascular disease but in a Danish cohort study (Schroll and Larsen 1981) this was not confirmed. Diabetes, hyperlipidaemia and thiazide diuretics may all influence urate levels and all are more common in patients with peripheral vascular disease. This association may therefore, just be secondary to these risk factors.

It is important to note that the presence of more than one of the above risk factors exponentially increases the risk of the development of atherosclerosis (Gordon and Kannel 1972). If a patient with no risk factors has a relative risk of 1 then with one risk factor their risk of IC increases three fold, two risk factors, it increases nine fold and three risk factors, twenty fold (Hughson et al. 1978).

NATURAL HISTORY OF CLAUDICATION

MORTALITY

Several epidemiological studies have revealed the profile of the natural history of IC. Work by O’Riordain and O’Donnell (1991) demonstrated that mortality after two, five and eight years was 8%, 23% and 40% respectively, corresponding to an annual death rate of 5%. Of those who died 44% died of cardiac related causes and 22% of cerebrovascular disease. One particularly interesting finding from multivariate analysis of this data was that the risk

factors of smoking, hypercholesterolaemia, age and sex, had no predictive value on mortality. However, when patients ankle brachial pressure index (ABPI) was analysed, an ABPI of ≤ 0.5 was associated with death rates at two, five and eight years of 20%, 50% and 69% respectively, compared to an ABPI of ≥ 0.5 where the death rates were 5%, 16% and 24% at the same time points ($p < 0.005$). In a multi-centre study of 1969 claudicants performed by Dormandy and Murray (1991) the above findings were confirmed with an annual death rate of 4.3%. Univariate analysis of ABPI of ≤ 0.5 again revealed it to be strongly predictive of mortality ($p < 0.001$), whereas hypertension, smoking and diabetes were not predictive. In a third study published the following year Criqui et al. (1992) demonstrated that although the traditional risk factors discussed above are crucial in the development and early progression of atherosclerosis they are not influential in the final stages of the disease. This study also demonstrated that claudicants have a 3.1-3.6 times increased risk of mortality compared to non claudicants and if cardiac disease was present then this relative risk rose to 5.6 times.

PROGRESSION TO CRITICAL ISCHAEMIA

Criqui et al. (1992) described IC as a relatively benign condition with only 30% of their study group progressing to critical ischaemia after eight years of follow up (an annual progression rate of 3.5%) and 66% of claudicants improving or remaining stable. This study was preceded by that of O'Riordain and O'Donnell (1991) again with 66% remaining stable and 34% deteriorating and Rosenbloom

et al. (1988) 59% stable or improved and 41% worse. Bloor (1961) has illustrated the magnitude of the mortality among claudicants in a six year follow up of 1476 patients in which five times as many patients died as underwent amputation. This demonstrates that the risk of death for claudicants is far higher than the risk of developing critical ischaemia and in the light of the Simvastatin study (SSSS 1994) our efforts should be directed at both investigating and treating their ischaemic heart disease and modifying their risk factors as this has been demonstrated to retard progression of atherosclerotic lesions.

MANAGEMENT OF THE CLAUDICANT

The message from our previous discussion is that the most useful part of management of IC is the investigation of the patients cardiac and carotid artery status. Risk factor modification will certainly reduce the likelihood of progression to critical ischaemia, but has no bearing on mortality.

The therapeutic strategy for patients with claudication depends on the age of the patient, the degree of handicap and the local practices of the surgeon in charge of the patients' care. Interventions range from a program of exercise, drug therapy, angioplasty and bypass surgery. The treatment options should be discussed with each individual and tailored to their needs and finally patients should be reassured that the risk of limb loss due to atherosclerosis is small provided they give up smoking.

NON INVASIVE THERAPY

STOP SMOKING

Smoking should be actively discouraged; cessation slows atherosclerotic progression and walking distance may improve (Radack and Wyderski 1990).

EXERCISE

In 1898 William Erb recognised the therapeutic benefit of exercise for patients who had claudication and subsequently several studies from Scandinavia have demonstrated the benefit of an exercise program (Larsen and Lassen 1966 and Ekroth et al. 1978). The initial claudication distance can improve two to three times during the first year of training but after the first year little further benefit is likely to be derived (Andriessen et al. 1989).

DRUG THERAPY

Low dose aspirin should be considered for all patients with IC. The risk of cardiac and cerebrovascular events is reduced by regular antiplatelet therapy (Antiplatelet Trialists' Collaboration 1994). Good control of other risk factors is desirable, such as hypertension in order to reduce the risk of stroke. There is

now evidence accumulating in favour of lowering serum cholesterol in order to improve walking distance. One study has demonstrated both reduction in plaque size in femoral arteries and improvement in severity of disease one year following commencement of aggressive lipid reduction regimens compared to “usual care” (Duffield et al. 1983) and the Scandinavian Simvastatin Survival Study Group (1994) conclusion that there is a reduction in cardiac mortality and morbidity from lipid lowering treatment in patients with ischaemic heart disease provides a strong rationale for intensive treatment of hyperlipidaemia. Also, Simvastatin has been shown to arrest and reverse the progress of coronary atherosclerosis measured by angiography (Kroon et al. 1996) and Lovastatin treatment has demonstrated the same effect when examining the carotid arteries (using Duplex doppler) (Hodis et al. 1996).

The role of vasodilators and so-called vasoactive drugs that modify blood flow characteristics is contentious. Capillary flow to the leg under ischaemic conditions is controlled mainly by the influence of local metabolites and ischaemic vascular beds are probably maximally dilated during exercise. A vasodilator may therefore, have a preferential effect on vessels that supply non ischaemic tissue and divert (“steal”) blood away from the ischaemic area. This may explain why pure vasodilators such as nifedipine and captopril do not improve walking distance in claudication. Some drugs, originally introduced for their vasodilatory actions also have effects on blood constituents and could improve flow. One example is oxypentifylline which alters white and red cell deformability and stickiness, lowers plasma fibrinogen and decreases platelet aggregation. Naftidrofuryl is claimed to promote the production of high energy

phosphates in ischaemic tissue and to reduce platelet aggregation. Clinical trials of these agents have rarely been rigorously controlled and provide little reliable evidence to support their use. Pooled data from the largest studies indicate a modest improvement in pain free walking distance compared to placebo, but the effect is usually too small to be clinically important (Porter et al. 1982 and Leheret et al. 1994).

Vasodilators have no place in the routine management of IC but for patients with rest pain and gangrene who are not suitable for reconstruction prostacyclin analogues have been shown to reduce amputation rates (UK Severe limb ischaemia group 1991).

INVASIVE THERAPY

The patient who has such disabling claudication that he/she is unable to work or care for themselves, or who presents with critical ischaemia (rest pain, ulceration and or gangrene), requires more invasive therapy. This comprises percutaneous transluminal angioplasty, or bypass surgery to revascularise the limb, with either autologous vein or prosthetic material. Prior to any intervention the extent and location of the causative lesion(s) needs to be identified using angiography.

ANGIOPLASTY

Percutaneous transluminal angioplasty was first described by Dotter and Judkins in 1964. They used coaxial catheters but the technical limitations of their equipment prevented widespread acceptance and use until Gruntzig and Hopff (1974) introduced the double lumen balloon catheter. This established angioplasty as an acceptable technique in the treatment of vascular stenoses. Balloon inflation results in splitting of the intima, plaque fracture and stretching of the vessel allowing reperfusion of a previously ischaemic area. The technique has been used to treat stenoses in the coronary circulation, subclavian, iliac, femoral, mesenteric and renal arteries.

The effect of angioplasty on the vessel wall is obviously injurious and therefore a potent stimulus for MIH and restenosis. This has limited the success of the procedure and also stimulated much work into MIH prevention over the last 20 years (See Chapter 2).

The results of angioplasty depend on the length and location of the lesion being treated. Aorto-iliac stenoses have an immediate success of 96% and five year patency of 90%, but iliac occlusions are less suitable. However, stenting may play an important role in the management of iliac occlusions, especially in poor surgical candidates (Rees et al. 1989). Superficial femoral artery lesions less than 2 cm in length have an 85% initial success rate with the long term results for stenosis superior to those of occlusions: at five years this is 70% when the lesion was a short segment stenosis, or 55% if originally an occlusion. The results for

popliteal artery angioplasty are similar to those of the superficial femoral artery (Rutherford et al. 1984).

New techniques available to the interventional radiologist are developing rapidly. Laser assisted angioplasty burns the atheroma inside the artery and was initially hailed as an effective therapy for stenotic atheromatous disease. The long term results showed it to be worse than balloon angioplasty but this therapy still has a role for long occlusions (Bauer et al. 1991). Atherectomy devices which contain high speed revolving cutters to core out lesions were introduced in 1988 (Simpson et al.). This device has a side window that is positioned up against the plaque which is then removed and the tissue stored in the device. This is ideal for focal eccentric lesions and the technique has a two year patency of 83-90% when used in iliac and femoral disease. Newer systems incorporate a cutting tip as opposed to a cutting side port that allows occluded vessels to be tackled. The most recent technology to become available to the vascular radiologist is the stent. This is mainly used to keep open recurrent stenoses or dissections, but their use does not prevent the re-accumulation of atheroma or the development of myointimal hyperplasia (MIH) at either end of the stent. These devices have been employed in both iliac and femoral arteries with 87% and 57% patency at one year. The use of atherectomy devices, lasers and stents is attractive because of the perceived less interventional nature of their insertion. This has to be weighed against their high restenosis rate (Satiani et al. 1993) and until this comes down bypass surgery will be the treatment of choice for extensive disease and longer occlusions. A study from Whyman et al. (1997) casts doubt on the lasting benefit of angioplasty for mild to moderate

claudication, with no difference between the angioplasty group and controls, after two years, in terms of patient reported maximum walking distance.

BYPASS SURGERY

Following the success of pioneering experiments by Carrel and Guthrie in 1906 using Vena Cava as a graft onto the Aorta and animal arterial homografts in the femoral artery, the technique for vein grafting for arterial repair spread throughout Europe and to the United States. However, technical problems particularly sepsis, prevented it being adopted widely until the 1950's. By this time surgical equipment and training had improved and the anti-coagulant heparin was available to counter the problems of thrombosis. Angiographic imaging had advanced to allow accurate location of lesions suitable for vein grafting and in 1949 Kunlin described the end to side anastomotic technique allowing the first use of the long saphenous vein in femoropopliteal grafting for the relief of ischaemic symptoms in the leg. This was adopted by Linton and Darling (1962) who, using reversed saphenous vein, reported an 86% success rate at up to 6 years after surgery. In 1968 Favaloro reported his vein graft technique for the revascularisation of the coronary circulation.

This early work demonstrated the feasibility of vein grafting and by 1972 there appeared no limit to the effectiveness of bypass surgery (Evans and Bernhard 1972), but work by Szilagyi et al. (1979) and Dardik et al. (1982) raised doubts about the overall benefit of the procedures, especially when revascularising the

crural vessels. They reported an increasing number of above knee amputations (~60%) for failed bypass surgery. So the indications for bypass surgery had to be more clearly defined and patients carefully selected for distal bypass or primary amputation. Careful assessment of the inflow vessel, and the run off are crucial to the success of reconstruction, particularly the latter which can usually be well imaged with modern digital subtraction angiography. Distal bypass surgery should on the whole be reserved for patients with critical limb ischaemia, or with severely disabling claudication (Second European Consensus Document 1991).

Once the decision has been taken to perform a bypass the next problem to be addressed is what is the best conduit to be used? In above knee femoropopliteal bypass there seems to be little difference between Polytetrafluoroethylene (PTFE) and vein as a conduit (Rosen et al. 1986 and Bergan et al. 1982). This has led many surgeons to adopt a policy of using PTFE in the first instance in the above knee segment and reserving the saphenous vein for further reconstruction that may be necessary at a later date. Below knee reconstructions provide a more clear cut picture with graft patency for vein being far superior to PTFE alone (62% Vs 30% at 3 years) (Wolfe et al. 1991). The reason for this is thought to be compliance mismatch predominantly at the distal anastomosis. However, the advent of the distal anastamotic interposition Miller collar (Miller et al. 1984), Taylor patch (Taylor et al. 1992) and St. Mary's boot (Tyrrell and Wolfe 1991), have all improved the patency of PTFE femoro-crural bypass to ~50% at 3 years. This is of crucial importance especially in patients undergoing

reconstruction who have had vein used for previous reconstructions in the leg or coronary circulation.

The experimental evidence suggests that these vein interposition techniques optimise the mechanical properties of saphenous vein and protect small arteries from anastomotic distortion. Hence the MIH develops in the venous interposition graft where the vessel is more capacious so protecting the recipient vessel. These benefits do not occur with direct PTFE-artery anastomoses (Tyrrell et al. 1990).

Finally, in selecting vein for the bypass should this be reversed or in-situ? The original technique of vein bypass described by Linton was done using reversed vein (to prevent the venous valves from impeding blood flow down the graft). The in-situ technique was described by Hall in 1962, the perceived benefit being derived from the fact that the natural change in diameter of the vein matched that of the artery which would allow better haemodynamics and easier anastomoses between size matched vessels. In addition less dissection of the vein was required thus preserving the vein wall blood supply from the vasa vasorum and hence reducing traumatic loss of endothelium. The main disadvantage of this method was the time consuming and tedious need for removal of individual valves. Advocates of the technique overcame the problems by developing methods to render the valves incompetent: Severing with blunt needles (Bellman and Kovamees 1966), breaking with a bull nosed stripper (Connolly and Harris 1965) and most recently, cutting with valvulotomes (Hall 1964, Mills et al. 1976 and Leather et al. 1984).

Despite good arguments in favour of the in situ technique the fact remained that Linton and Darling's (1962) original good results and subsequently those of Lo Gerfo et al. (1977) and Grimley et al. (1979) were all obtained using the reversed vein method. There was therefore, little impetus for surgeons to change technique.

Experimental work has not resolved the issue, in fact it has exposed flaws in both techniques. Studies from dogs show that endothelial loss is increased along with medial VSMC replacement with fibrosis in reversed vein technique (Buchbinder et al. 1981 and Wyatt 1964). However, endothelial and VSMC injury still occur to a significant degree in the in situ method (Sayers et al. 1992). The theoretical haemodynamic benefit has not been supported experimentally either (Beard et al. 1986).

In a randomised clinical study comparing graft outcome between the two different techniques there appears to be no appreciable difference in patency rates (Harris et al. 1993) and it is therefore a matter of the personal preferences of the surgeon.

VEIN GRAFT STENOSIS

HISTORY

Following the initial enthusiasm that accompanied the vein bypass graft, reports of graft failure began to appear (Breslau and DeWeese 1965, Beebe et al. 1970, Downs 1971 and De la Rocha et al. 1973). In a key piece of work by Szilagyi et al. (1973), studying the natural history of autologous vein transplants, vein grafts were followed up over ten years. In this comprehensive study they found a 21% incidence of vein graft stenosis, detected using angiography, which was used to follow up all the grafts. All the stenotic lesions underwent histological examination. They also identified the adverse effects that vein graft stenoses have on long term graft patency. Subsequently the incidence of vein graft stenosis has variously been reported as 28% (Bandyk et al. 1988 and Moody et al. 1989), 17% (Taylor et al. 1990) and 37% (Buth et al. 1991), with 75% occurring in the first year (Berkowitz et al. 1981). In the study by Taylor et al. (1990), all stenoses had been detected by 1 year but the time period between each surveillance episode was much shorter than in Berkowitz's (1981) study, in which it was not clear if the grafts were examined during the first year after operation. Femoropopliteal and femorocrural vein bypass graft failure continues to be a problem of considerable magnitude, with failure rates reported at 20%-50% at 5 years (Buda et al. 1976, DeWeese and Rob 1977, Maini et al. 1978 and Bandyk et al. 1987). In a study from Brewster et al. (1983) looking at 555 femoropopliteal grafts it was found that 65% of all failures had occurred in the first year and 79% by the end of the second year.

The causes of vein graft failure are clustered into three groups. In the immediate postoperative period and up to about thirty days, grafts fail because of technical factors such as twists, compression, inappropriate selection of patients with poor outflow, inadequate graft diameter and technical errors in the construction of anastomoses; this occurs in 5%-10% of all grafts and at six weeks 95% of grafts are still patent. Between one month and eighteen months the graft wall thickens as an adaptive response to the increased pressure and wall stress that the graft is subjected to upon transposition from the venous to the arterial circulation. Stenoses develop at anastomoses or along the graft and cause low flow which will result in spontaneous thrombosis. These stenoses when examined are fibrous, contain smooth muscle cells and matrix and are as a result of myointimal hyperplasia (MIH) (see next chapter); this occurs in approximately 25% of all grafts, leaving a patency of 70% after 1 year. Beyond eighteen months the graft may fail as a result of progression of the atherosclerotic process either in the graft or native vessels at a rate of about 2% per year leaving a patency of 50% at five years. In addition, grafts may fail as a result of aneurysmal dilatation, degeneration or infection and rarely as a result of spontaneous thrombosis due to underlying haematological disorder (Whittemore et al. 1981). If vein graft patency is to be improved then our understanding of the development of MIH stenoses must improve, as they are responsible for nearly 80% of all graft failures (Brewster et al. 1983 and Taylor et al. 1990).

Only a few studies have analysed the factors associated with vein graft stenosis. Sladen and Gilmour (1981) found no association between stenoses and

hypertension, smoking, age, gender, presenting symptoms and outflow artery. Berkowitz et al. (1989) demonstrated an increased incidence of stenoses in longer (more distal) grafts: above knee 8%; below knee 12% and tibial 18%). Also in reversed vein grafts the most common site for stenoses was within 4 cm of the proximal anastomosis 40% (Berkowitz et al. 1992). Mills et al. (1993) reported the majority of stenoses developing in the juxta-anastomotic position both proximally and distally. The conclusion from this data was that haemodynamic factors may be responsible in the aetiology of these lesions at these locations although there is little supporting evidence at this time.

Approximately 20% of all grafts develop a stenosis and 80% will occur in the first year, these lesions are usually localised (<2cm) (Moody et al. 1992, Berkowitz et al. 1992 and London et al. 1993). Frustratingly, the majority of graft stenoses are not associated with the redevelopment of ischaemic symptoms nor reduced peripheral pulses (Grigg et al. 1988) so some form of graft surveillance is necessary to detect these stenoses.

SURVEILLANCE

The concept of pro-active intervention was founded in the early 1980's by Whittemore et al. (1981) and Berkowitz et al. (1981) when they independently determined that correction of a failing graft delivers better long term patency than intervention following graft failure. This was also later borne out in work by Moody et al. (1990) which demonstrated a 15% increase in graft patency

when comparing surveillance and intervention with an historical control group. Recently Lundell et al. (1995) demonstrated prospectively that early aggressive post-operative surveillance (three monthly) resulted in 25% higher 1 year assisted patency compared to annual surveillance (and secondary patency). As only 30%-50% of failing grafts present with symptoms of recurrent ischaemia that are detectable (Wolfe et al. 1987) a reliable method of surveillance of infra-inguinal vein grafts is essential if stenoses are going to be detected before they resulted in vein graft failure. During the 1980s various modes of surveillance have been employed: the one minute treadmill test described by Laing and Greenhalgh (1980) using ankle brachial pressure indices alone would only detect 50% of asymptomatic stenoses. Intravenous digital subtraction angiography can be used and is effective at picking up asymptomatic stenoses (Wolfe et al. 1987) but it is demanding on staff and patients and gives no direct haemodynamic information. With the availability of duplex scanning in the early 1980s there was much enthusiasm among vascular surgeons in search of a surveillance tool. With technical refinement and combination of parameters used to determine the presence of stenoses, by the early 1990s a highly sensitive and specific tool had indeed evolved. Using colour flow imaging to determine the average peak systolic velocity (less than 45 cm/sec being indicative of a stenosis) proposed by Bandyk et al. (1988) and the velocity ratio ($V_2:V_1 > 2$ being indicative of a stenosis) proposed by Grigg et al. (1988), Taylor et al. (1990) established a test for graft stenosis that was 100% sensitive and 98% specific. Subsequent work by Cheshire et al. (1993) has shown that surveillance is effective both in financial and limb salvage terms. Mills et al. (1995) also demonstrated that grafts with normal early duplex Doppler scans exhibited a

low incidence of stenosis development, however, an early focal flow abnormality developed into a high grade stenosis in 50% of patients. At this point in time surveillance is our most powerful weapon against graft failure. Recently the value of surveillance has been questioned in a study from Golledge et al. (1996) which demonstrated that although surveillance improved graft patency, there was no improvement in limb salvage.

MYOINTIMAL HYPERPLASIA.

HISTOPATHOLOGY

In 1908 Carrel first noted that vein grafts placed in the arterial circulation will universally develop some degree of thickening along their length, subsequently known as MIH, but it is where this process is excessive that it becomes the major factor causing graft failure (Campbell et al. 1981, Szilagyi et al. 1973 and Fuchs et al. 1978). Any form of vessel wall injury, whether accidental or deliberate, induces a wound healing response that includes proliferation and migration of smooth muscle cells and endothelial regeneration.

MIH is characterised by substantial thickening of the intimal layer of the vessel wall. The thickened intima is composed of fibrocellular connective tissue of variable cellularity, surrounded by extracellular matrix of collagen and ground substance. In the early stages of MIH the cellular component predominates; in the mature lesion, more extracellular matrix is evident. MIH may coexist with atherosclerosis, which as previously described is more complex, with more elements of cellular proliferation, inflammation and fibrosis, as well as regions of necrosis and accumulation of intracellular lipid (Dilley et al. 1988).

MIH is a necessary component of healing following vascular injury (Clowes et al. 1986). However, when the process becomes excessive it will cause stenosis of the new conduit. Surgical endarterectomy and angioplasty will also result in the development of MIH: 34% of aorto-iliac endarterectomies have significant recurrent stenoses detectable by angiogram at one year (Szilagyi et al. 1964). This failure rate has been largely responsible for the procedure going out of favour. Following carotid endarterectomy there is a 9%-19% incidence of high grade recurrent stenoses (defined as over 50% reduction in luminal area, usually measured by Duplex Doppler) within one year of surgery (Shumway et al. 1987 and Healy et al. 1989). However, the symptoms produced seldom warrant reintervention.

Development of recurrent stenosis by MIH is the major limiting factor in the long term success following percutaneous transluminal angioplasty, with coronary angioplasty attended by 25%- 40% restenosis rate within six months (McBride et al. 1988 and Popma et al. 1992), peripheral arterial angioplasty resulting in 28-50% restenosis in the femoropopliteal segment and 7-10% restenosis in the iliac segment (Rutherford 1994 and 2nd European Consensus Document on Chronic Critical Limb Ischaemia 1991). Bowles et al. (1988) also reported MIH (histologically confirmed) causing arterial stenosis 4 months following balloon catheter embolectomy. The newer technique of atherectomy appears to provoke an equally high MIH response in the vessel wall of 30-60% (Bowles et al. 1988 and McGurrin et al. 1991).

MIH stenoses are not uniformly distributed throughout the site of vascular injury, with thickening of the vessel wall notable around anastomoses, particularly at the downstream site (LoGerfo et al. 1983). With the standard end to side vein graft anastomosis the intimal thickening is most marked at the floor of the native vessel opposite the graft, the suture line and the toe of the graft (Bassiouny et al. 1992).

PATHOPHYSIOLOGY

Ultrastructural analysis of the MIH lesion reveals it to have a cellular basis. The predominant cell type is the vascular smooth muscle cell (VSMC). Cells with an appearance similar to fibroblasts are also seen, as are cells of indeterminate appearance. However, in the uninjured artery there are no fibroblasts present in the media or intima, vascular VSMCs making up virtually all of the media, with the occasional representation in the intima (Woolf 1978 and Chamley-Campbell et al. 1979). There is believed to be a continuum of histological appearances between the fibroblast and the VSMC, implying that these are not distinct cell types, but can differentiate from a common origin in both directions (both are mesodermal in origin) (Sottiurai and Batson 1983a). There is support for this argument from cell culture studies in which cells can be made to differentiate into either a contractile (VSMC-like) phenotype or a synthetic (fibroblast-like) phenotype (Chamley-Campbell and Campbell 1981). It is likely that these observations could account for the historic confusion that has arisen around the term graft “fibrosis” and the appearances of MIH. The proliferating VSMC in

the developing MIH lesion adopts a synthetic phenotype similar to the fibroblast and secretes collagen and extracellular matrix which ultimately form the bulk of the mature lesion. The graft stenosis represents the mature MIH lesion. The source of the proliferating VSMC is from the media underlying the lesion. Following vein graft insertion the medial VSMC adopt a synthetic phenotype and produce extracellular matrix. These VSMC initially migrate into the intima and proliferate (Dilley et al. 1988 and Forrester et al. 1991). The stimulus for the process is arterial trauma resulting in endothelial denudation and exposure of the basement membrane and media to platelets. The platelets will degranulate and this results in the release of peptide growth factors that are both chemotactic (Bell and Madri 1989 and Madri et al. 1989) and mitogenic (Ross and Glomset 1973) for VSMCs. This stimulates the VSMCs to migrate and proliferate in the intima. There is evidence of autocrine and paracrine stimulation of VSMC, allowing the process to continue well beyond the resolution of the original platelet stimulation (Fritze et al. 1985 and Golden et al. 1990). Only 30% of the medial VSMC respond to the injurious agent by proliferation and migration (Clowes and Schwartz 1985 and Clowes et al. 1989) as assessed by labelled thymidine uptake by the VSMC in the balloon injured rat carotid model. Though this is not necessarily applicable to humans.

The relative importance of the contribution of the various peptide growth factors in the development of the MIH lesion in humans is not yet clear. However, PDGF transcripts have been found by in situ hybridisation in atherosclerotic plaques (Wilcox et al. 1988), and PDGF like mitogen is secreted by cultured VSMC derived from anastamotic hyperplastic lesions (Birinyi et al.

1989). PDGF is also expressed by cultured human venous endothelial cells (Limanni et al. 1988) and intimal cells soon after placement of PTFE grafts into the circulation (Golden et al. 1990). This all lends weight to the concept that endothelial injury and exposure of the media to platelets with subsequent growth factor release is the precursor to MIH. There is however, evidence from the rat model to the contrary, demonstrating that following induced thrombocytopaenia the injured vessel will still develop a MIH lesion (due to VSMC proliferation) although attenuated. This prompted the authors to propose that platelets may be more involved in stimulating VSMC migration than proliferation (Fingerle et al. 1989).

The balloon injured rat carotid model of MIH has been extensively studied especially by Clowes. His group have shown that maximal VSMC proliferation occurs by three days following injury with VSMC proliferation increasing to 20% from the normal level of 1% (Clowes et al. 1983 and Clowes and Schwartz 1985). Continued proliferation is dependent on endothelial coverage; in injured segments covered by endothelium proliferation returns to baseline within eight weeks after injury, but remains elevated if segments remain chronically denuded of endothelium.

The point at which the lesion causes luminal narrowing is earlier than in humans, at about two weeks and is caused at least initially by VSMC contraction. The intimal thickening becomes measurable by one week but is not maximal until four to twelve weeks following injury. The number of VSMCs does not increase after the third to fourth week and the increasing bulk of the

lesion is accounted for by increasing extracellular matrix deposition. The three major connective tissue proteoglycans are chondroitin sulphate, dermatan sulphate and heparan sulphate and they make up the early matrix components which in the fully mature lesion are replaced by collagen (Swedberg et al. 1989).

Further evidence for the ultrastructural modulation that occurs in the MIH lesion is provided by two studies; one looking at changes in the coronary arteries following balloon dilatation and a second at the changes in the carotid artery following endarterectomy. In Clagett et al. (1986) and Sterpetti et al. (1989) studies, lesions removed from reoperated carotid arteries were histologically examined revealing that early restenotic lesions (< 24 months) were predominantly cellular with abundant VSMCs. Later lesions (>24 months) were almost completely acellular except for foam cells, calcification, haemosiderin and neovascularisation; all features of atherosclerosis. The study by Nobuyoshi et al. (1991) in post-mortem sections of coronary arteries that had previously been angioplastied showed in the 20 lesions examined within 6 months of angioplasty that there were abundant proliferating VSMCs and extracellular matrix. Between 6 months and 2 years the lesions were predominantly collagen matrix with some VSMCs. However, beyond two years as in Sterpetti's (Sterpetti et al. 1989) study the lesions were indistinguishable from atherosclerosis.

The two processes of atherosclerosis and MIH are inextricably linked, sharing aetiological and developmental characteristics. The response to injury hypothesis put forward initially by Virchow (1856) and subsequently refined by

Ross and Glomset (1973) has proved popular and spurred the explosion in interest in the biology of VSMC.

FACTORS INFLUENCING MIH DEVELOPMENT

It is fairly certain that peptide growth factors cause cellular proliferation and migration and hence MIH. However, what is not clear is why some individuals develop vein graft stenoses and not others? It therefore seems logical to assume that other factors are influencing the development of MIH.

MECHANICAL

Mechanical factors, in particular those related to blood flow, have been implicated in the aetiology of MIH in animal models. The normal laminar flow pattern in arteries produces a relatively slower moving layer next to the artery wall called the boundary layer and the force this layer exerts on the vessel wall is known as the shear stress. In conditions of slow flow or turbulence, flow in the boundary layer may be static or reversed. This is known as boundary layer separation, as blood constituents and platelets are in prolonged contact with the artery wall. This has been proposed as a possible mechanism for arterial wall pathology.

The phenomenon of low flow causing low shear stress and being associated with the development of MIH was demonstrated initially by Rittgers et al. (1978) using the dog femoro-femoral crossover bypass, although this correlation was weak. Subsequently Ojha et al. (1990) used a plexiglass model of an end to side 45° anastomosis and demonstrated a complex helical flow pattern at normal arterial pressure with pulsatile flow. The areas of localised low shear stress roughly corresponded to the sites of predilection for MIH; the anastomosis toe and the floor of the recipient artery opposite the anastomosis. Bassiouny et al. (1992) modelled casts derived from rabbit arteries that had previously been operated on and identified flow patterns by passing microbeads through these models. A relationship between low shear stress and turbulent flow was demonstrated, with the location of these forces corresponding to sites of known predilection for MIH: floor of recipient vessel and suture line. Sottiurai and Batson (1983a) looked at adult human carotid bifurcations obtained at autopsy and also demonstrated intimal thickening and atherosclerosis developed largely in regions of relatively low wall shear stress. It appears that the endothelial cell is responsible for sensing the change in shear stress (Kraiss et al. 1991) and then either releases or stimulates the release of peptide growth factors (Hsieh et al. 1991). Conversely, high shear stress has been proposed as a causative factor in the production of MIH with the vein graft adapting to this higher shear by wall thickening (Kohler et al. 1989).

Graft compliance has been postulated as a possible aetiological factor in MIH development, the compliance mismatch at the anastomosis being responsible for creating turbulent blood flow. This is less likely to be a problem with vein

grafts than with PTFE where the mismatch is greater (Lo Gerfo et al. 1979). The haemodynamic forces discussed above make a strong case for anastamotic MIH but do not explain the occurrence or location of intra-graft stenoses.

VEIN GRAFT FACTORS

INTRINSIC

To further answer the question of graft body stenoses it is important to consider pre-existing venous disease. Sanchez et al. (1991) pointed out that morphological changes seen in the intimal layer of long saphenous vein and MIH in vein grafts were very similar and speculated that they may be related. In a subsequent study looking at the morphology of the vein wall he found (Marin et al. 1994) that veins with pre-existing thick or calcified walls or hypercellular intima (spindle cells greater than five cell layers thick) were at ten times greater risk of developing vein graft stenoses that would lead to graft failure, when compared to non thickened vein grafts. Davies et al. (1994a) have also found that thickened vein (associated with reduced vein wall compliance) was associated with an increased rate of stenosis. However, recent work from Varty et al. (1996) has shown that preoperative vein morphology has no influence on the subsequent development of stenoses.

EXTRINSIC

The response to injury model is widely accepted as the explanation for MIH and atherosclerosis and so mechanical vein graft injury must be considered as an aetiological factor in the development of discrete intra-body-vein graft stenoses. In an elegant study by Moody et al. (1992) all sites where vein graft trauma had occurred were marked with surgical clips: clamp injury, venotomies, valve division, tributaries and residual valve cusps. There was no relationship between the development of subsequent vein graft stenoses and the site of the clips. This human data contrasts with the vast body of evidence derived from animal models which have shown that balloon injury (Clowes and Karnowski 1977), air drying or clamping of vessels results in the development of MIH lesions.

THE INFLUENCE OF CONVENTIONAL RISK FACTORS FOR ATHEROSCLEROSIS ON MIH.

The most obvious candidates for stimuli for the development of MIH would be the traditional risk factors discussed for atherosclerotic vascular disease. This data is readily accessible in virtually all series of patients studied.

In reviewing factors responsible for restenosis following coronary angioplasty McBride et al. (1988) found no correlation between restenosis risk and age,

cigarette smoking, hypertension or hyperlipidaemia. However, diabetes was associated with a slightly higher incidence of restenosis.

In examining 109 femoropopliteal vein grafts Whittemore et al. (1981) found no significant correlation with cigarette smoking, diabetes, hypertension or coronary artery disease and the occurrence of vein graft failure in their patient population.

Stonebridge et al. (1994) discussed the adverse effects of smoking on vascular reconstruction and concluded that to continue doing so after reconstructive surgery was highly likely to result in graft failure.

Yusuf and Probstfield (1989) point out a consistent relationship between late failure of coronary vein graft and elevated serum cholesterol or low density lipoprotein- cholesterol (LDL). However, it is important to note that these failures are late and the likely cause is progression of the atherosclerotic lesions and not MIH. This finding was echoed by Campeau (1987) and Solymoss et al. (1988) who demonstrated angiographic changes in vein grafts of patients with elevated levels of LDL 10- 12 years following surgery and again this was attributed to atherosclerosis not MIH.

Shah and Amin (1992) reported reduced levels of high density lipoprotein- cholesterol (HDL) in 28 patients who developed restenosis after coronary angioplasty compared with controls who did not develop restenoses. In a similar study by Colyvas et al. (1992) looking at 20 patients who had developed

stenoses following carotid endarterectomy they were found to have significantly reduced HDL levels compared to controls. Cuming et al. (1993) examined restenosis rates after one year in patients undergoing carotid endarterectomy and found smoking was associated with an increased restenosis rate. In the same study, hyperlipidaemia was not an influential factor in men, but was positively related to stenosis in women. By contrast, elevated serum HDL in conjunction with a low LDL is considered protective against the development of atherosclerosis (O'Connor et al. 1990 and Rouffy et al. 1985).

Szilagyi et al. (1973) looked at 377 vein grafts in the lower limb and found no relationship when comparing elevated total serum lipids, triglycerides and cholesterol with graft atherosclerosis. Smoking and diabetes were not related either.

In the multi-centre study conducted by Wiseman et al. (1989) in Britain, elevated serum fibrinogen levels and smoking were predictors of graft failure. Interestingly in this study elevated serum cholesterol and serum low density lipoprotein-cholesterol (LDL) were correlated with improved graft patency! However, the evidence for fibrinogen being an important risk is getting stronger as both Cheshire et al. (1996) and Hicks et al. (1996) have demonstrated a link between elevated serum fibrinogen and graft stenosis.

Bergamini et al. (1991) have reported a series of 361 consecutive in situ vein bypasses to popliteal and tibial arteries, in which neither smoking nor diabetes were considered determinants of either primary or secondary patency.

Lipoprotein (a) (LP(a)) is an LDL like lipoprotein whose increased concentration is related to an increase in the risk of atherosclerosis (Scanu and Fless 1990). LP(a) has been shown to increase the migratory capacity of the bovine VSMC (Kojima et al. 1991). Cheshire et al. (1996) also demonstrated raised levels of LP(a) in patients who developed stenoses in infra-inguinal vein grafts compared to those controls who did not.

The overall picture is that the classical risk factors for atherosclerosis are neither consistently nor strikingly related to the failure of vascular procedures. The inference can be drawn that these factors are not risk factors for restenosis and are probably unrelated to the aetiology and development of MIH. Nevertheless, it would be wrong to conclude that no risk factors exist. Some factor(s) must distinguish the roughly 30% of patients who (re)stenose from the 70% who do not. In the realm of vein grafting there is anecdotal data from DeWeese and Rob (1977) of individuals failing several grafts sequentially; however there is stronger evidence from Dimas et al. (1992) who found the rate of repeated restenosis following coronary angioplasty at 57% almost double that of the primary rate of 30%. Earlier work from Holmes et al. (1984) in an epidemiological survey clearly indicated that a particularly strong risk factor for coronary restenosis after angioplasty was a previous restenosis, not necessarily at the same site.

MODELS OF MIH

MIH is a universal feature of healing of the vessel wall after injury and it is not surprising that there is a huge variety of models described. Animal models of MIH reproduce the response of the arterial wall to injury or the response of a graft, vein or prosthesis, to placement in the circulation. These models have provided most of the present knowledge of the sequence of events that follow vascular injury, however there is no adequate model of progressive, stenotic MIH as seen in humans.

Imperato et al. (1972) used a dog carotid artery as a free graft between the abdominal aorta and the vena cava or femoral artery and vein. Clowes and Karnowsky (1977) describe injury to the rat carotid initially using a balloon catheter then using hot air to dry the intima. Murday et al. (1984) described an interposition graft of external jugular vein placed to the rabbit common carotid artery. In dogs Morinaga et al. (1987) used reversed femoral vein to restore continuity of a 5 cm excised segment of femoral artery and Fillinger et al. (1989) used a loop of 6 cm Poly-tetraflouroethylene (PTFE) between femoral artery and vein. In pigs Schwartz et al. (1992) delivered tantalum coils into pig coronary arteries using an angiography catheter. In baboons Golden et al. (1990) used a PTFE prosthesis inserted into the thoracic aorta. Itoh et al. (1990) found MIH significantly complicating their model of PTFE prostheses placed as vena caval replacement in rabbits.

One of the major criticisms of all this work in animal models of vascular injury is that they do not reproduce the stenotic type of MIH seen in humans. In particular dogs have much less intimal response than other animals and rarely develop stenosis after vascular injury. Rabbits have usually been fed hypercholesterolaemic diets to encourage a stenotic response; they usually develop a generalised lipidosis with deposition in the reticuloendothelial system. These animals do develop a stenotic lesion but as these animals are fed an artificially high fat diet to induce extremely high serum cholesterol levels their metabolic state hardly parallels the typical disturbances of metabolism seen in humans with atherosclerosis (Muller et al. 1992).

Animal models are not useful for screening a large number of agents as possible therapies against MIH as there are considerable operative morbidities associated with some of the models and this would not justify screening large numbers of agents, many of which must be ineffective. Additionally, the pharmacology and pharmacokinetics of many agents differ substantially between species and a therapeutic response in an animal model cannot be assumed to translate into favourable human response.

Angelini et al. (1991) has popularised a human saphenous vein organ culture model developed by Pederson and Bowyer (1985) and demonstrated tissue viability throughout 14 days in culture. They have demonstrated an intimal hyperplastic response composed largely of extracellular matrix which is only sparsely cellular. This is in contrast to the *in vivo* lesion which is densely cellular during the first 14 days. Saphenous vein can only be cultured for 14 days as

after this the tissue deteriorates. Also, very high concentrations (30%) of FCS are required to maintain the endothelial cells. At this concentration VSMC do not proliferate (*vide infra*). This is supported by their own experiments that show by removing the intima the hyperplastic response can be abolished. This contrasts with the *in vivo* model where endothelial denudation promotes the MIH response (Clowes et al. 1986).

The models described above probably demonstrate a normal adaptive response of initially normal vessels to injury. MIH in humans is almost certainly a maladaptive response of diseased vessels and as such is likely to be considerably different from the models described above. As the small mammal model does not do justice to this human problem I have turned to a human cell culture system to further investigate the biology of MIH.

PREVENTION OF MIH

Morinaga et al. (1987) showed that established MIH may undergo some degree of spontaneous regression over time. However, the return or deterioration of symptoms implies the presence of significant vessel narrowing, which may be severe enough to allow thrombosis to occur. At this point revision surgery or angioplasty are required.

Treatment for MIH therefore, should focus on prevention and identification of high risk groups to receive prevention. Ideally this should have little toxicity

and be administered to all patients undergoing vascular reconstruction. Most work to date has focused on surgical technique and prophylactic drug prevention.

TECHNICAL FACTORS

Improvements in surgical technique, due to greater experience and increased familiarity with perioperative care, have increased the patency rate of coronary vein grafts in the Montreal Heart Institute from 74% to 85% at one year. However, the one month patency rate remained unchanged at 90%. There was a modest increase in overall graft patency during the study period however, as the end point was graft occlusion and as grafts were not studied for stenosis it is hard to attribute this improvement to a reduction in MIH.

The influence of specific technical factors on the development of MIH in experimental models is conflicting. Carrel and Guthrie noted in 1906 that the vessel wall thickening was most pronounced at the point where the dog 's jugular vein had been traumatised by vascular clamps. By contrast, Storm et al. (1975) showed that after stripping the adventitia off the dog femoral vein and distending it to 200 mmHg there was no effect on the development of MIH after grafting. A clinical study by Moody et al. (1992) failed to show any increase in vein graft stenosis at sites where vein graft trauma had occurred. Angulation of the anastomosis in the dog femoral vein to iliac artery fistula model or aortocoronary saphenous vein model has no effect on the incidence of MIH,

neither does producing a stenosis in the run off vessel beyond an arterio-venous fistula (Bond et al. 1976 and Breyer et al. 1976).

DRUG TREATMENTS

The majority of human clinical trials have examined angioplasty site restenosis or graft patency at any time between 1 month and 18 months as end points.

Aspirin and dipyridamole have not demonstrated any improved patency rates over placebo in a multi-center trial from London and Birmingham. 549 patients were randomised to receive aspirin 300mg and dipyridamole 150mg twice daily against placebo. Cumulative graft patency was 72%, 62% and 60% at 1, 2 and 3 years respectively in the placebo group whilst in the aspirin group 78%, 70% and 61%. None of these were statistically different (McCollum et al. 1991).

Chesebro et al. (1984) reported an improved early and 3- 12 month patency rate (statistically significant 8% improvement) using aspirin and dipyridamole. The authors themselves state in the discussion that the regimen is unlikely to have impressive effects on graft failure caused by MIH and attribute the favourable results entirely to the effects on thrombosis.

There is a modest 10 % improvement with antiplatelet agents which appear to improve coronary vein graft patency at early periods (4 months or less) (Yusuf

and Probstfield 1989) as well as over the period in which MIH is a prominent cause of failure (3months to 1 year).

The data from vein graft studies does not appear to be echoed by post angioplasty restenosis trials. These demonstrate that aspirin/ dipyridamole and ticlopidine are of no benefit in preventing restenosis (Ip et al. 1991 and Schwartz et al. 1988). A trial of GR32191B a platelet anti-aggregatory agent also appeared to be ineffective with no prevention of restenosis either (Serruys et al. 1991).

When prosthetic graft trials are studied, there is evidence from Green et al. (1982) that 12 month patency for above knee PTFE femoro-popliteal grafts is improved if patients are given aspirin and dipyridamole preoperatively. However, 1 and 2 year patency was not improved when patients were given both aspirin and dipyridamole, in similar studies by Kohler et al. (1984).

The evidence seems to support antiplatelet agents as improving early graft patency by preventing thrombosis but they do not appear to influence the development of MIH.

Systemic anticoagulation does not provide a clear benefit either. One hundred and nineteen patients were randomised to receive either phenprocoumon or placebo after femoro-popliteal bypass. Although there was a slight survival benefit for the anticoagulated patients there was no improvement in graft patency (Kretschmer et al. 1988). In another earlier study by Evans and Irvine

(1966) looking at femoro-popliteal patency with warfarin there was no improvement in graft patency. Analysis of coronary vein graft patency in two smaller studies show no benefit in the anticoagulated group at 2 and 6 months (Gohlke et al. 1981 and Panteley et al. 1979). Anticoagulation following coronary angioplasty does not effect restenosis either (Ip et al. 1991).

There is little evidence to suggest that heparin prevents MIH in humans. In a study by Ellis et al. (1989) where patients were randomised to either 18- 24 hours of heparin post angioplasty or placebo (both groups received anticoagulation with 10,000- 15,000 units of heparin during the procedure), there was no significant difference in rate of restenosis between the two groups: 41.2% in heparin treated and 36.7% in the placebo group. Follow up was by angiography at six months (60% of randomised patients). However, in a study by Edmondson et al (1994) low molecular weight heparin for 3 months was found to reduce the risk of graft occlusion three fold compared to patients receiving aspirin and dipyridamole but this was only in a sub-group analysis of patients undergoing limb salvage surgery.

Fish oils (Eicosapentanoic acid), first observed to produce increased bleeding times in Greenland Eskimos, have subsequently been studied for their potential beneficial effects on the cardiovascular system. Dehmer et al. (1988) and Bairati et al. (1992) reported a significantly reduced rate of restenosis 3- 4 months following coronary angioplasty in patients receiving a diet rich in fish oils. However, in two studies looking at fish oil versus corn oil (Grigg et al. 1989) and fish oil versus olive oil (Reis et al. 1989) no difference in restenosis rates was observed. In the largest of such trials to date, a supplement of 8 g/day of

omega-3 fatty acids failed to prevent the usual high rate of restenosis after PTCA (Leaf et al. 1994).

Many clinical trials are currently ongoing, with the objective of reducing the rate of restenosis following coronary angioplasty. In fact, Popma et al. (1992) reported ongoing studies of 11 different agents some of which have now reported. Those that show benefit are unfortunately flawed in design.

A trial looking at the lipid lowering agent lovastatin showed a reduced incidence of coronary restenosis (Sahni et al. 1991). However, the numbers were small, the trial was not double blinded, follow up angiography was only carried out on 50% of randomised patients and follow up was only 2 months. Similarly, the European verapamil angioplasty study reported that the drug reduced the incidence of coronary restenosis (Hoberg and Kubler 1991). In this study the incidence of control group restenosis was almost twice that normally expected and the drug was only found to be effective in one of the two patient groups. Controlled clinical trials have failed to show a benefit after angioplasty: the restenosis rate was not significantly influenced by either nifedipine or diltiazem at lower doses, 90 mg three times per day. Another trial used a higher dose of diltiazem (mean 329 mg per day) in a larger number of patients, but again the result was negative. By contrast, diltiazem 180 mg per day was reported more recently to significantly reduce restenosis, and high-dose verapamil treatment was similarly successful, but only in a subgroup of patients with stable angina pectoris at the time of primary successful angioplasty (Hoberg 1995).

The putative PDGF antagonist trapidil was reported as being beneficial (Okamoto et al.1992) but this only looked at a small study group and had short follow up. In addition, patients received nifedipine, a drug with potential to inhibit VSMC proliferation (Nilsson et al. 1985).

A recent randomised study comparing radiotherapy delivered via a coronary angiography catheter versus placebo, in a group of patients who had previously undergone angioplasty (with or without stenting for an atherosclerotic lesion) and developed a restenosis, demonstrated a significant reduction in the incidence of restenosis in the radiotherapy treated group (17% v 54%) (Teirstein et al. 1997). This line of research may offer hope in the management of this problem, however, the incidence of restenosis in the untreated group was higher than might normally be expected, at 54%, so these results must be treated with some caution.

The disappointing results of human trials can be largely attributed to the eagerness of clinical investigators to tackle an important problem. Agents have been used in these studies based on extrapolation of animal data without considering inter species variability and that animal models do not reproduce human vascular restenosis.

EXPERIMENTAL TREATMENT FOR MIH

The effect of drug treatment on MIH, rather than overall graft patency has been extensively studied in small mammal models.

The first effective agent described was heparin, used in anticoagulant doses in rats, following MIH induced by carotid injury (Clowes and Karnofsky 1977). The effect was shown to be unrelated to heparin's anticoagulant properties when non anticoagulant heparin was found to be equally as effective in the prevention of MIH (Guyton et al. 1980). Orally administered cyclodextrin sulphate (a non anticoagulant synthetic heparin mimic) was found to inhibit restenosis in hypercholesterolaemic rabbits (Herrman et al. 1993) (See chapter 4).

Topically applied heparin in slow release medium (poly vinyl alcohol) when delivered to the injured rat carotid produced a reduction in MIH without systemic anticoagulant effect (Okada et al. 1989). However, when using heparin bonded stents in pig coronary artery MIH was not prevented, in fact, there was a greater intimal response in the stented group compared with the angioplastied group (Jenkins et al. 1995).

The antiplatelet drugs which in man appear to have an effect on early graft patency have conflicting results in animal MIH. Murday et al. (1984) found no

evidence of effect in the vein interposition model in the rat carotid. However, McCann et al. (1980) in the primate model and Metke et al. (1979) in the dog found that MIH was reduced 6 weeks following surgery.

Prazosin inhibited MIH in the rabbit aorta balloon injury model (O'Malley et al. 1989) and cilazapril reduces MIH in the rat carotid balloon injury model (Powell et al. 1989). In general agents which lower blood pressure also inhibit VSMC proliferation and agents causing a rise in blood pressure stimulate VSMC proliferation (Jackson and Schwartz 1992).

It is important to note that the doses of these drugs were considerably higher than would normally be used in man and the effect of cilazapril is not consistent between the species. It will effectively reduce MIH in rats and rabbits; less so in the guinea pig and not at all in monkeys and pigs (Clozel et al. 1991, Lam et al. 1992 and Hanson et al. 1991). When it is effective it appears to work through angiotensin converting enzyme inhibition, as an angiotensin II AT1 receptor antagonist was equally effective at inhibiting MIH (Prescott et al. 1991).

The Cholesterol synthetase inhibitor terbinafine has been effective at inhibiting MIH in the rat carotid balloon injury model (Nemecek et al. 1989) and lovastatin an HMG-CoA reductase inhibitor which lowers serum cholesterol will inhibit MIH in the rabbit carotid balloon injury model (Gellman et al. 1991).

Studies by Sarris et al. (1989) of hypercholesterolaemic dogs given fish oil rich diets, show that the effect of these compounds on vein graft stenosis is unrelated to lipoprotein levels, platelet aggregability or prostaglandin-thromboxane metabolism.

Athymic nude rats demonstrated reduced MIH in response to vein grafting in the femoral artery segment (Saenz et al. 1991), but developed significant MIH after carotid balloon angioplasty, no different to euthymic rats heterozygous for the nude gene (Ferns et al. 1991b). Cyclosporin A has been reported to inhibit MIH in rats (Jonasson et al. 1988 and Stoltenberg et al. 1994) However, cyclosporin is nephrotoxic, immunosuppressive and raises blood pressure. Other immunosuppressive agents such as azathioprin and cyclophosphamide have failed to inhibit MIH in rabbit carotids (Chervu et al. 1989). However corticosteroids have been reported to be successful at preventing MIH in the rabbit carotid balloon injury model at equivalent doses to that used in man (Colburn et al. 1992 and Chervu et al. 1989).

Leukotrienes, particularly interleukins and monocyte derived cytokines may be involved in VSMC proliferation (Bonin et al. 1989). Interleukin 1 probably stimulates mitogenesis through the autocrine production of PDGF-AA (Raines et al. 1989). Interferon gamma, a T-cell derived cytokine, does inhibit in vivo intimal thickening in the rat (Hansson et al. 1991).

Angiopeptin is a synthetic peptide analogue of somatostatin and has been reported to inhibit MIH in the rat vein graft and rabbit arterial balloon injury models (Calcagno et al. 1991). This compound has also been evaluated in a randomised clinical trial to assess its potential for preventing restenosis after coronary angioplasty and the results show a significant reduction in the incidence of restenosis in the angiopeptin treated group compared to controls (12% v 40%). Clinical events and late lumen loss also were significantly reduced after angiopeptin (Eriksen et al. 1995).

An ornithine decarboxylase inhibitor has been shown to reduce MIH in the balloon injured rabbit carotid (Endean et al. 1991). Ornithine is the rate limiting precursor in the synthesis of polyamines, intracellular cations that are involved in cell differentiation and proliferation. Hexamethylenebisacetamide, an inhibitor of the enzyme ADP phospho-ribosyltransferase (a key enzyme in differentiation (loss of the heavy chain myosin of the VSMC)), will also selectively inhibit VSMC proliferation in cell culture (Grainger et al. 1992).

Nitric oxide (NO) donors, in addition to their vasodilator effect, decrease platelet aggregation and inhibit vascular smooth muscle cell proliferation (Lablanche et al 1997). Using a periadventitial delivery system Chaux et al. (1998) reported reduced intimal hyperplasia in veins implanted in the arterial circulation. The mechanism of action is not entirely clear, but the reduction cannot be explained on the basis of decreased cell proliferation alone. In a

prospective multi-center trial 700 stable coronary patients scheduled for angioplasty received direct NO donors (infusion of linsidomine followed by oral molsidomine) or oral diltiazem. Although, the NO donor group had a modest improvement in the long-term angiographic result after angioplasty there was no effect on clinical outcome (Lablanche et al 1997).

The object of investigation of the above drug therapies most frequently and conveniently used has been arterial injury in small mammals. As you can see from this list of compounds, most drugs available in the cardiovascular armory have been tried, in animals, in order to prevent the development of MIH with considerable success. However, in nearly all cases when these compounds have been tried in man they have been ineffective. Because the small mammal model does not do justice to this human problem I have turned to a human cell culture system to further investigate the biology of MIH.

CHAPTER 3

THE HUMAN VASCULAR SMOOTH MUSCLE CELL IN CULTURE.

INTRODUCTION

The vascular smooth muscle cell (VSMC) is pivotal in the formation of MIH. The VSMC migrates from the media to the intima and proliferates, then secretes extracellular matrix which forms the bulk of the MIH lesion. The VSMC in culture may provide a model in which agents can be tested for their antiproliferative and anti-migratory effects. Also studies of the mechanism of VSMC growth may contribute to our understanding of MIH and the early stages of atherosclerosis (Ross and Glomset 1973).

In 1914 Champy and Laqueur and Lewis and Lewis (Murray and Kopech 1953) first demonstrated the feasibility of maintaining and growing cells from a variety of animal tissues. Since the late 1950's most of the work on VSMCs in culture examined the uptake, synthesis and metabolism of lipids by these cells and also VSMC proliferation and matrix synthesis (Chamley-Campbell et al. 1979).

In vivo the VSMC may change between two phenotypes, contractile and secretory. At one end of the spectrum the contractile VSMC is composed to a greater extent of contractile elements. The most predominant is the “thin” filament which is composed of actin and tropomyosin. “Thick” filaments are made up of myosin and lie along the long axis of the VSMC and are surrounded by the “thin” elements. These insert into dense bodies which are themselves closely associated with “intermediate” filaments. These are composed of Skeletin and form the cytoskeletal framework of the VSMC. At the synthetic end of the phenotypic modulation the VSMC contains very few myofilaments and has abundant free ribosomes and rough endoplasmic reticulum. Although VSMC are capable of division in the contractile form the majority observed in mitosis are of the synthetic phenotype.

The majority of studies on VSMC culture have been performed using the substrate attached explant method although the VSMCs can be cultured using the enzyme disaggregation and free floating explant method. I have used the former method, stripping the adventitia off the media and scraping away the intima, then cutting the remaining media into ~ 2mm square pieces and placing them into flasks containing sufficient nutrient medium to prevent drying but not too much that would allow them to float. Explants usually attach to the substrate within a few days and the VSMC start to migrate out from the explant.

During preparation of the vessel wall for culture care is taken to remove all intima and media, as endothelial cells and fibroblasts respectively may

contaminate the cultures. These cells can be distinguished from VSMC by their growth characteristics. The VSMC in culture is typically spindle shaped and adopts an “hill and valley” growth pattern due to the relative density and sparsity of the cells. “Hills” can be up to 15 cell layers thick and “valleys” as thin as one layer (Chamley-Campbell et al. 1979).

Fibroblasts adopt a variable shape and form concentric “whorls” in culture. Endothelial cells usually adopt a polygonal appearance with raised centrally located nuclei and usually only form monolayers.

Endothelial cells can be distinguished in culture by staining with fluorescein labeled antibodies to factor VIII related antigen and VSMC can be clearly distinguished from endothelial cells and fibroblasts by staining with antibodies to smooth muscle actin and myosin.

VSMC proliferation requires phenotypic modulation from contractile to synthetic with concomitant loss of myosin. Once confluent in the culture flask, the VSMC reverts to its contractile phenotype after approximately 1 week, although, the content of myosin is never as high as originally. Clearly during the proliferative phase the VSMC is very similar to the fibroblast . However, immunofluorescent staining with antibodies to actin and tropomyosin is not usually diminished in the synthetic phenotype making them distinguishable from fibroblasts.

Cultured VSMCs have been shown to synthesize 70% type I and 30% type III collagen. Elastin and proteoglycan synthesis in the form of heparan sulphate, chondroitin sulphate, dermatan sulphate and hyaluronic acid have also been demonstrated

Early attempts at culture of VSMC from human arteries were reported to produce inconsistent results (Gimbrone and Cotran 1975) probably due to contamination with endothelial elements. Subsequently more successful attempts have been reported using facial and thyroid artery (Hosang and Rouge 1989), uterine artery (Fager et al. 1989), superficial temporal artery (Aoyagi et al. 1991) and abdominal aorta from transplant donors (Bjorkerud 1991).

More recent attempts have shown it is possible to culture VSMC from saphenous vein derived from patients undergoing vascular and cardiac surgery (Chan et al. 1993 and Munro et al. 1994) the very patients that develop clinically relevant MIH lesions. Also it has been possible to culture VSMC from the MIH lesions itself, when removed at the time of revision surgery (Gilbertson et al. 1992 and Munro et al. 1994). These cells maintain a remarkable degree of consistency of responses in culture from passage to passage.

CELL CULTURE CONDITIONS

For cell culture to proceed successfully one requires conditions of strict sterility, and control of humidity, temperature and pH and a nutrient medium suitable for growth. The cells are grown in sterile incubators maintained at a constant 37°C in an environment with 5% CO₂ and added HEPES buffer to maintain optimal pH. The VSMC requires a basic nutrient medium, such as Dulbecco's Modification of Eagle's Minimal Essential Medium (DMEM) to fulfill their metabolic requirements and an additional factor to stimulate cell replication. The standard agent used is foetal calf serum (FCS), which is derived from heat inactivated (at 56°C) whole blood, to inactivate complement, and sterilised by passage through a 200µm filter. There is considerable batch to batch variability, so they must be tested before use. The same batch of FCS was used in comparative experiments.

VSMC can survive for several days in a quiescent state in medium containing concentrations as low as 0.4% FCS (Chamley-Campbell et al. 1979). They can also be maintained for a similar time in serum free medium supplemented with insulin, transferrin, selenium and ascorbate (Libby and O'Brien 1983). Serum at a concentration 5-20% is necessary to stimulate cell growth in culture. The constituents of serum that are necessary for cell growth have not been identified, but platelet degranulation products which are released during clotting play an important role. Serum derived from platelet poor plasma has lost almost all its growth promoting power when compared to serum derived from platelet rich plasma (Ross and Glomset 1973). This led to the discovery of

platelet derived growth factor, although serum contains a variety of other growth factors.

PEPTIDE GROWTH FACTORS

The exact mechanisms whereby VSMC proliferation is initiated, controlled, reduced and eventually suppressed are not fully understood. Growth factors which are polypeptides can regulate the proliferation of endothelial cells, the proliferation and migration of VSMCs and the secretion of extracellular matrix. Quiescent VSMC are contractile cells that are well differentiated and characterised by an abundance of contractile proteins, predominantly actin and myosin but little rough endoplasmic reticulum (Owens and Thompson 1986). Once activated they become synthetic and are less well differentiated with an abundance of endoplasmic reticulum and with the capacity to synthesize extracellular matrix and growth factors. In order to put their role in the development of the MIH lesion into perspective, their structure, origins, properties and mechanism of action will be described.

In recent years much has been learned about the molecular and cellular events initiating the repair process in healing wounds. From these studies, growth factors that are integral to various phases of wound healing and evidence has also accumulated that growth factors play an important role in the normal repair mechanism of the vessel wall.

Injury to the endothelium as a result of balloon catheter dilatation or thrombectomy, endarterectomy or constructing an anastomosis precipitates two crucial events: adhesion and activation of platelets and adherence of monocytes. Within minutes of injury, platelets and leukocytes adhere to the exposed sub-endothelial collagen. Platelets aggregate and release pro-thrombotic substances in addition to growth factors such as PDGF and TGF- β (Bowen-Pope et al. 1989). Monocytes migrate into the sub-endothelial layer, differentiate into macrophages and release their growth factors.

Endothelial cells migrate to cover the denuded area within hours of the injury. Proliferation is usually evident by 24 hours (Reidy 1985) and the distance between the margins of the denuded area determines the extent of endothelial migration and proliferation (Clowes et al. 1986). Jawien et al. (1992) has demonstrated that, in the balloon catheter injury model in the rat, re-endothelialisation ceases at approximately six weeks despite the presence of extensive denuded areas. Basic FGF is readily demonstrable in replicating endothelial cells and when administered systemically facilitates endothelial re-growth (Lindner et al. 1989, 1990 and 1992). These observations suggest that FGF plays a role in the restoration of endothelial integrity.

Simultaneously VSMC in the media proliferate and migrate through disrupted portions of the internal elastic lamina into the intima. Approximately 20- 40% of VSMC enter their growth phase and synthesize DNA within 30 hours of injury and continue to proliferate for 7 to 14 days after which proliferation spontaneously ceases (Clowes and Schwartz 1985).

The intent of these events is to repair the damaged vessel wall and the release of PDGF, EGF, FGF and IGF results in the proliferation of different cell types in the vessel wall. PDGF stimulates VSMC to migrate and proliferate to repair a defect in the media or restore normal architecture of the intima. EGF and IGF facilitate the activity of PDGF and stimulate reconstruction of the endothelial monolayer as does FGF.

For reasons not yet fully understood normal cellular reparative processes become dysfunctional in MIH, with the same growth factors that mediate repair of the vessel wall now acting to promote proliferation and migration of VSMC out of proportion to the degree of injury.

SIGNAL TRANSDUCTION

Signal transduction is the process whereby cell surface receptors convert external stimuli into appropriate intra-cellular responses. There are basically two ligand operated receptor mechanisms responsible for the signal transduction: first, ligand-modulated release of a secondary intra-cellular mediator (G protein-coupled receptors) and second, ligand-regulated enzyme activity (tyrosine kinase coupled receptors). Guanine nucleotide regulatory proteins (G proteins) are a super family of guanosine 5'-triphosphate (GTP) binding proteins that range from the monomeric to the heterotrimeric forms. The heterotrimeric G proteins are intrinsic membrane bound proteins that act as trans-membrane signal transducers and they can inhibit and activate adenylate

cyclases and phospholipases to produce second messengers. These functionally related G proteins are also involved in protein synthesis, microtubule assembly and oncogene function (Gilman 1987, Neer and Clapham 1988, Kelvin et al 1989) all of which are important in VSMC proliferation and hence MIH.

Platelet Derived Growth Factor (PDGF)

PDGF is a 28 to 35 kDa cationic glycoprotein that acts as a potent mitogen for several types of mesenchymal-derived cells including vascular VSMC (Bobik and Campbell 1993). It was first described by Ross and Glomset (1973) as a soluble substance released from aggregating platelets and is produced by platelets, monocytes, vascular endothelial cells and VSMCs. It is composed of two polypeptide chains: A chain (14-18 kDa) and B chain (16kDa). Because these two chains are encoded by different genes on separate chromosomes (Betsholtz et al. 1986) and production of each chain is independent of the other, it can exist in three different isoforms PDGF-AA, PDGF-BB and PDGF-AB each subunit being linked by disulphide bonds (Bobik and Campbell 1993). When released the PDGF molecule acts on a dimerised receptor which is composed of two distinct subunits: α and β to initiate an intra cellular signaling cascade discussed above (Seifert et al. 1989 and Bowen-Pope et al. 1989). The α subunits can bind both A and B chains of PDGF, whereas, β subunits can bind only B chains. Therefore, of the three dimeric combinations $\alpha\alpha$, $\beta\beta$ and $\alpha\beta$: $\alpha\alpha$ can bind PDGF-AA, PDGF-AB and PDGF-BB; $\alpha\beta$ can bind PDGF-AB and PDGF-BB and $\beta\beta$ can only bind PDGF-BB. As mentioned above all three receptor isoforms are

proposed to act as intrinsic membrane tyrosine kinases. In human platelets 70% of “platelet derived” PDGF is the AB isoform and the remaining 30% is the BB isoform (Hammacher et al. 1988). PDGF is present only transiently in the circulation (half life of less than two minutes), implying that it is rapidly cleared under normal circumstances. In the circulation the concentrations are in the ng/ml (picoMolar) range (Bobik and Campbell 1993) and *in vitro* PDGF isoforms are able to stimulate tritiated thymidine uptake in human vascular VSMC in the same range (Hughes et al. 1995). Production of the B chain of PDGF is directed by the *c-sis* oncogene, found on the long arm of chromosome 22 (Josephs et al. 1984). The A chain gene is located on chromosome 7.

VSMC from injured vessels express A and B chain genes for PDGF but secrete only A chain (Lindner 1995). However, only the β receptor subunits are upregulated in the VSMC, suggesting that cells may not be able to respond to PDGF in an autocrine fashion (only $\beta\beta$ can bind PDGF-BB) (Majesky et al. 1990). This begs the question: what is the source of PDGF in the development of the MIH stenosis? It is known that platelets on the luminal surface release mainly the BB isoform thus, providing an agonist for the newly formed VSMC PDGF β receptors (Bowen-Pope et al. 1989).

Further evidence for the role of exogenously derived PDGF BB being important in the genesis of the MIH lesion comes from the observations that insertion of the PDGF BB gene into balloon injured porcine arteries results in the development of MIH (Nabel et al. 1993a), infusion of the PDGF BB gene promotes and accelerates VSMC migration but has little effect on proliferation

(Jawien et al. 1992) and infusion of anti-PDGF antibody has been shown to inhibit MIH thickening by up to 40% with little effect on the degree of VSMC proliferation (Ferns et al. 1991). Recent work from Clunn et al. (1997) has demonstrated that PDGF BB appears to change its function dependent on the concentration; being chemo-attractant at low concentrations with little effect on stimulation of VSMC proliferation and changing to being highly stimulatory to proliferation and inhibitory to chemotaxis at high concentration.

If the platelets and platelet factors are so important in VSMC migration, what triggers the first wave of proliferation? and furthermore as the VSMC is unresponsive to its own PDGF, what sustains the progression of the MIH lesion? Other factors produced by damaged VSMC and endothelial cells must be involved.

Fibroblast Growth Factors (FGF)

FGFs are a family of heparin-binding growth factors that can be synthesised by both vascular endothelial cells and VSMCs (Gospodarowicz et al. 1987 and Klagsbrun and Edelman 1989). First described as a factor (derived from bovine pituitary) that could promote cell division in fibroblasts (Gospodarowicz et al. 1974) FGF is also known to be a potent mitogen for vascular VSMC as well as promoting endothelial cell proliferation and migration. Two forms have been identified: acidic and basic, each a 154 amino acid, single chain polypeptide with a molecular weight of approximately 15 kDa with a high affinity for

heparin (Burgess and Maciag 1989). There is 55% homology between acidic and basic FGF. However, basic FGF is much more active than acidic *in vitro*. Likewise the FGF receptor exists in two forms: a 125 kDa and 145 kDa protein. The acidic form binds to the 125 kDa receptor and the basic to the higher affinity 145 kDa receptor. Following binding the FGF receptor complexes are internalised (Rusnati et al. 1993).

Unlike other growth factors, FGF exists largely as an intracellular peptide, which can be deduced from the absence of signal peptide in its gene sequence (Burgess and Maciag 1989, Rifkin and Moscatelli 1989). This suggests that FGF may function as an autocrine/ paracrine repair factor that is stored intracellularly and only released extracellularly by cell injury or death (Vlodavsky et al. 1991).

Although FGF is predominantly an intracellular peptide it is found in significant quantities in the extracellular matrix produced by endothelial cells, where it is bound to heparan sulphate within the basement membrane. This acts to potentiate its mitogenic action and prevent degradation of the FGF (Saksela et al. 1988). There is strong evidence for its involvement in MIH. Following endothelial damage and denudation FGF is released from the matrix by the action of heparinases from neighbouring neutrophils, T-lymphocytes and platelets (Rogelj et al. 1989). The basic FGF receptors are up regulated following balloon injury (Casscells et al. 1992) and are expressed on the surface of injured vascular VSMC that concentrate basic FGF on the surface of the cell (Gospodarowicz et al. 1987, Klagsbrun and Edelman 1989). Also, infusion of

FGF has been shown to promote the development of MIH stenoses but only when the endothelium has been removed, with intact vessels unresponsive to basic FGF (Lindner et al. 1990, Edelman et al. 1992 and 1993), whereas, infusion of antibodies to basic FGF have been shown to inhibit MIH development in some animal models (Lindner and Reidy 1991). Although FGF appears to be important in initiating the VSMC proliferation seen in MIH it is still unclear if it is required in continuing VSMC proliferation (Olson et al. 1992).

By contrast to basic FGF, acidic FGF infusion is capable of promoting vascular repair with decreased MIH in a dose dependent manner (Bjornsson et al. 1991). To further confuse the issue, insertion of acidic FGF genes into the arterial wall promotes both angiogenesis and MIH (Nabel et al. 1993b).

Epidermal Growth Factors (EGF)

EGF was first described in 1962 by Cohen and is a single chain polypeptide comprising 53 amino acids with a molecular weight of 6kDa (Cohen 1962 and Taylor et al. 1972). EGF is present in and released from activated macrophages, as well as a number of other cell types (Tekatani and Oka 1983, Sprugel et al. 1987 and Strandjord et al. 1994). *In vitro* EGF is a mitogen for ectodermal and mesodermal cells, including fibroblasts and VSMCs (Carpenter et al. 1975). The EGF receptor is a 17 kDa protein with a receptor bound tyrosine kinase. Binding of EGF to its receptor results in autophosphorylation of the receptor-bound tyrosine kinase (Cohen et al. 1982). At least part of the activity of EGF appears

dependent on the internalisation of the EGF receptor complex and its transport to the nucleus (Schlessinger et al. 1978). It is noteworthy that EGF acts synergistically with other peptide growth factors particularly PDGF either as a competence factor or as a promotor depending on the prevailing culture conditions (Reilly et al. 1987). Although much is known about the *in vitro* role of EGF little is known about its role *in vivo*, particularly with regard to the development of MIH.

Insulin like Growth Factor (IGF1)

IGF denotes a class of proteins whose basic structure is similar to pro-insulin (~50% homology) but whose functions are quite different. Rather than stimulating glucose transport, IGF1 stimulates proliferation of vascular VSMC (Bornfeldt et al. 1994). IGF is a basic protein composed of 70 amino acids and contains three disulphide bridges. Both IGF and PDGF can induce the synthesis and release of IGF binding proteins, which may serve as either reservoirs of IGF in the vessel wall or act as physiological inhibitors of VSMC proliferation (Drakenberg et al. 1990 and Gianella et al. 1992). Following vessel wall injury the expression of IGF mRNA is increased 9 fold mainly in the VSMC located in the vessel media (Cercek et al. 1990). IGF1 may also participate in the MIH lesion by increasing the number of PDGF receptors in VSMCs (Pfeifle et al. 1987) IGF appears to mediate its action via tyrosine kinase linked cell surface receptors (Ververis et al. 1993)

Transforming Growth Factors (TGF- α and TGF- β)

The transforming growth factors were named for their ability when combined to promote the growth of normal rat kidney fibroblasts independent of a matrix. Otherwise, they are similar in name only. In fact, TGF- α is much more similar to EGF than it is to TGF- β . TGF- α is a 50 amino acid polypeptide with a molecular weight of 5.6 kDa, the gene for which lies on the short arm of chromosome 6 (Fowler et al. 1993). Although it shares only 35% homology with EGF, through similarities in its three dimensional conformation it occupies the same receptor as EGF. In fact, no distinct receptor for TGF- α has been found. Not surprisingly its actions are similar to those of EGF. TGF- α promotes migration of endothelial cells and increases their proliferation *in vitro* and it may be that its role is in repair of damaged vessel wall (Schultz et al. 1991).

TGF- β has distinctly different properties than TGF- α , it is also a polypeptide with molecular weight 25 kDa and consists of two identical chains linked by disulphide bonds (Roberts and Sporn 1985). It exists in homodimeric (β_1 and β_2) and heterodimeric ($\beta_1\beta_2$) forms. It is secreted in latent form and is activated by the action of plasmin (Sperti et al. 1992). It is a mitogen for fibroblasts and VSMCs while it has significant inhibitory activity for PDGF dependent growth of both these cell types. TGF- β is secreted by macrophages, platelets, monocytes and VSMCs and is a potent chemoattractant for fibroblasts, VSMCs, monocytes

and macrophages (Childs et al. 1982, Assoian et al. 1983 and 1987, Wahl 1994, Postlethwait and Seyer 1995).

TGF- β can stimulate the proliferation of VSMC through a PDGF like intermediate while inhibiting the proliferation of endothelial cells (Majack et al. 1990). The receptor for TGF- β is present on many cell types and there are three different types with various binding affinities, type III being expressed by VSMCs (Nikol et al. 1992). Low concentrations of TGF- β induce the expression of the PDGF AA isoform which can stimulate VSMC proliferation. However, high concentrations of TGF- β will inhibit the production of the PDGF α subunit of the receptor thus acting to inhibit VSMC proliferation (Battegay et al. 1990).

TGF- β has a profound effect on the composition of the extracellular matrix secreted by the endothelial cell and VSMC and this in turn will have effects on the growth of the VSMC. TGF- β increases the synthesis of proteoglycans, collagen types I, II, III, IV & V, thrombospondin and elastin (Bobik and Campbell 1993). TGF- β can also influence matrix composition by controlling the activity and secretion of degrading enzymes such as plasminogen activator inhibitor-1 produced by VSMC (Reilly and McFall 1991).

In the study of human (re)stenosis higher levels of TGF- β mRNA has recently been detected by in situ hybridisation techniques in VSMC from atherectomy specimens compared to specimens from atherosclerotic plaques, saphenous vein and internal mammary artery lending support to its involvement in

restenosis but not stenoses developing *denovo* in vascular grafts (Nikol et al. 1992). TGF- β is unique among growth factors in that it may either promote or inhibit cellular proliferation, depending on the target cell type and the presence or absence of other growth factors in the local environment. Consequently, a central regulatory role has been ascribed to TGF- β in the maintenance and repair of the vessel wall although its role in (re)stenosis is as yet uncertain.

Angiotensin II (AII)

The renin angiotensin system is now recognised as crucially important in the maintenance of cardiovascular homeostasis. The components of these local renin-angiotensin systems are located primarily in the endothelium, but they have also been found in the VSMCs in the media (Dzau 1993).

AII is a vasoactive octapeptide formed from an inactive precursor, angiotensin I by the action of the enzyme angiotensin converting enzyme (ACE). ACE is a membrane bound dipeptyl peptidase. The local renin-angiotensin system has been implicated in directing the responses of the vessel wall to injury. The expression of angiotensinogen gene is upregulated maximally at 7 days in the media following vessel injury (Rakugi et al. 1994a) and local tissue ACE activity also correlates closely with the degree of MIH (Rakugi et al. 1994b). In the balloon injured rat carotid model, but not in pigs and primates, ACE inhibitors were able to inhibit both the extent of MIH if the injury if it was superficial (Powell et al. 1989) and this is associated with a dose dependent reduction in

tissue ACE activity. However, this response is dependent on the depth of the injury; ACE inhibitors are not effective at preventing MIH when the injury is deep (Miyachi et al. 1998).

Angiotensin II (Ang II) can bind to at least two different receptor subtypes: AT₁ and AT₂ (Griendling et al. 1994) and recent evidence suggests that both of these are involved in the MIH process. Firstly, the AT₁ receptor antagonist Losartan (MSD) significantly reduces MIH following balloon injury in the rat carotid (Kauffman et al. 1991) but not in the rabbit iliac (Powell et al. 1989). Secondly AT₂ antagonists infused locally at the site of injury will reduce MIH formation but not when systemically infused (Janiak et al. 1992). ACE inhibition was effective at preventing restenosis in hypercholesterolaemic rabbits and normal rats, but did not occur in clinically more relevant porcine models nor in large clinical trials. The critical analysis of present experimental findings on a beneficial influence on both the spontaneous and the progressive development of atherosclerosis indicates ACE-inhibition to be more likely to preserve or restore the function of an intact endothelium than to interfere with the complex reaction occurring after injury of an already affected blood vessel (Becker 1994).

Free Radicals

The endothelial cell can both produce and degrade oxygen free radicals (Davies and Hagen 1993). Increased intracellular concentrations of these radicals inactivate nitric oxide (NO) and can inhibit the production of prostacyclin

(PGI₂) in endothelial cells. Loss of the antiproliferative effects of NO and PGI₂ will lead indirectly to VSMC proliferation. In addition to this above effect, oxygen free radicals can induce *c-fos* and *c-myc* proto-oncogene expression each of which is associated with proliferation in VSMC (Rao and Beck 1992). Oxidative stress may also be important in restenosis (Schneider et al. 1993 and Blann et al. 1993) with levels of active free oxygen species increased in blood vessels in response to angioplasty of vein grafts in animals and free radical scavengers reducing the extent of MIH (Davies et al. 1994b and Hagen et al. 1992). Damage to the vasa vasorum causing medial ischaemia has also been shown to cause MIH (Barker et al. 1993) lending further weight to the evidence that hypoxia and oxygen free radicals are important in MIH. Most recently Rodes et al. (1998), using the antioxidant probucol, demonstrated a reduction in lumen loss and restenosis rate after balloon angioplasty in a patient group with small coronary arteries.

As part of my work I shall look at the effects of some of these growth factors in isolation on VSMC proliferation and migration (Chapters 7, 8 and 9).

THE ROLE OF HEPARINS IN VSMC PROLIFERATION

The proteoglycan heparan sulphate is part of the extracellular matrix found in the blood vessels and is a component of the basal lamina of both VSMC and endothelial cells (Fransson 1987). It is attached to the cell surface in two distinct ways: ionic bonding of the heparan side chains to surface receptors ; alternatively it may be attached to the cell membrane by intercalation of a hydrophobic region of the core protein directly into the lipid bilayer (Kjellen et al. 1980). Heparan sulphate has many biological effects in the vessel wall. It plays a role in M1H controlling the phenotype that VSMC express, inhibiting cell transit from G₁ to S phase of the cell cycle and binding a variety of growth factors.

The effect of heparan on VSMC phenotype was discovered by seeding enzyme dispersed cells at subconfluent density in primary culture and this resulted in a gradual decrease in their myosin content (Campbell et al. 1989). However, if the basal lamina is rapidly reconstituted by seeding the cells onto basement membrane containing heparan sulphate then they do not undergo this phenotypic modulation (Stadler et al. 1989). Plating freshly dispersed cells at confluent density will achieve the same effect. The contractile phenotype can also be maintained by placing sparsely seeded VSMC with a spatially separated feeder layer of confluent, myosin rich VSMCs or confluent endothelial cells (Chamley-Campbell and Campbell 1981). Both feeder layers are known to

produce large amounts of antiproliferative heparan sulphate (Fritze et al. 1985 and Castellot et al. 1981). Exposing sparsely seeded VSMC to an extract of glycosaminoglycans from myosin rich, aortic media and intima or by the addition of heparin will maintain the cells in the contractile phenotype (Chamley-Campbell and Campbell 1981). This effect can be abolished by adding heparinases (Campbell et al. 1992).

The method of action of both heparan sulphate and the closely related glycosaminoglycan heparin is not clear. Cell associated heparan sulphate can promote organization of intracellular actin filaments which has the effect of stabilizing cell morphology (Herman 1990). Hence, degradation of VSMC surface heparan, by prolonged enzyme action during dispersal, may initiate a change in VSMC phenotype through disorganization of actin filaments. However, the observation that low concentration trypsin alone (which releases heparan sulphate from the VSMC surface) does not induce phenotypic modulation, suggests that the glycosaminoglycan must be totally destroyed for this to occur (Campbell and Campbell 1991). The fact that heparin itself can prevent this phenotypic change in VSMC whose extracellular matrix has been degraded supports this view.

Both heparan and heparin bind to specific VSMC surface proteins and are internalised by endocytosis and degraded (Bienkowski and Conrad 1984, Schmidt and Buddecke 1988). A small fraction of the glycosaminoglycan is

transported to the cell nucleus where it has been implicated in regulation of growth (Fedarko and Conrad 1986, Castellot et al. 1985b). It is not clear if internalisation of heparan and heparin is involved in phenotypic modulation, however, the observation that myosin rich contractile VSMCs constantly internalize and degrade their own cell surface heparan suggests that this may be the case (Campbell and Campbell 1991).

The inhibitory effect of heparin on VSMC proliferation was first shown *in vivo* by Clowes and Karnowsky (1977). Upon heparin treatment the VSMC proliferation seen after endothelial denudation was almost completely prevented. This effect is independent of heparin's anticoagulant properties (Guyton et al. 1980).

The ED₅₀ for the antiproliferative action of heparin in rat arterial VSMC is in the range 1- 10 $\mu\text{g}/\text{mL}$ (Hoover et al. 1980). VSMC that are in the G₀ phase of the cell cycle are 50 - 100 times more sensitive to the antiproliferative action of heparin than VSMC that are proliferating at exposure (Castellot et al. 1981). The concentration of other glycosaminoglycans such as chondroitin and dermatan sulphate needs to be 50 to 100 (at least 500 $\mu\text{g}/\text{mL}$) times greater in order to achieve an antiproliferative effect similar to heparin at its EC₅₀. The smallest heparin fragment capable of inhibiting VSMC proliferation is a pentamer, whereas, di- and tetrasaccharides are inactive. The maximum antiproliferative action is achieved with a dodecamer and larger fragments (Castellot et al. 1986).

It appears that O-Sulphation or at least negative charge at the O position is required for antiproliferative activity in the VSMC (Castellot et al. 1984 and 1986 and Wright et al. 1989a). Heparan sulphate derived from endothelial cells appears to be 1000 fold better than heparin at inhibiting VSMC proliferation (Benitz et al. 1990 and Castellot et al. 1981).

Heparin appears to act mid to late G_1 approximately 4 hours before S phase in the cell cycle (Castellot et al. 1985a and Reilly et al. 1989). The G_0 to S transition can be divided into two phases: the competence phase, in which growth factors stimulate the cell to leave the G_0 state and become “competent” to traverse the rest of G_1 and the progressive phase, in which competent cells move through the rest of G_1 and enter S phase (Pledger et al. 1977). Heparin also inhibits a protein kinase C-dependent pathway for cell proliferation and suppresses the induction of c-fos and c-myc mRNA at a site distal to activation of the kinase (Wright et al. 1989b).

Heparan Sulphate can modulate growth by influencing the availability of growth factors within the vessel wall. Heparin has been shown to bind bFGF (Burgess and Maciag 1989) and this seems to protect it from degradation and also provides a reservoir of the growth factor on the cell surface (Folkman et al. 1988). TGF- β and PDGF also bind to heparan sulphate on the VSMC surface. The long A chain of PDGF binds to the glycosaminoglycan part of the molecule

temporarily inactivating it (Fager et al. 1992). Conversely TGF- β binds to the proteoglycan part of the molecule via the core protein (Andres et al. 1989).

VSMCs are characterized by their acute growth inhibition by heparin and heparan sulfates; however, the isolation of VSMCs which display greatly diminished sensitivity to the antiproliferative action of heparin (Chan et al. 1993) gives us an opportunity to study the mechanism of heparin's antiproliferative action in human VSMC. To date these mechanisms have remained elusive, but further study of human VSMCs may expand our understanding of cell growth regulation by heparin.

PROSPECTIVE CLINICAL STUDY.

INTRODUCTION

Why should it be that ~30% of grafts and angioplasties develop stenoses ? Studies have shown that classical risk factors for atherosclerosis are not related to the development of stenoses (Chapter 2). However, there do now appear to be some serum risk factors for individuals predisposing to the development of stenoses: patients with elevated serum levels of fibrinogen (Cheshire et al. 1996 and Hicks et al. 1996) and of lipoprotein (a) (Cheshire et al. 1996). However, at present, we are unable to predict pre-operatively who is likely to develop a stenosis and so our current ability to manage the stenosis relies on surveillance and the detection of a lesion before it results in graft failure. In the case of infra inguinal vein grafts this is done using duplex Doppler in conjunction with angiography in order to detect an established stenosis (Taylor et al. 1992, Cheshire and Wolfe 1993). Therefore, although surveillance is going to prevent some graft failures by allowing early reintervention with angioplasty or graft revision, this is in effect treatment rather than prevention.

Twenty years ago it was found that heparin inhibited VSMC proliferation, both *in vivo* (Clowes and Karnowski 1977, Guyton et al. 1980) and *in vitro* (Hoover et

al. 1980), a phenomenon that has also been demonstrated in the human VSMC culture model developed by Chan et al. (1993). In their experiments on the effect of heparin on VSMC proliferation, this group demonstrated a pronounced heterogeneity of responses between VSMC derived from different individuals. Of particular interest was the finding that the population of patients who had already developed graft stenoses possessed vein derived cells that were predominantly resistant to the anti-proliferative effects of heparin. Even more striking was the finding that vascular smooth muscle cells cultured from unaffected vessels in these same individuals shared this tendency to heparin resistance. Further work using this model has shown that the properties of cultured VSMC in response to heparin are consistent from passage to passage in the same cell line. It was also shown that VSMC taken from different sites along the same segment of saphenous vein (proximal, middle and distal) behave in a consistent fashion in response to heparin as do VSMC from paired vein and artery (Munro et al. 1994). This data raises fundamental issues: since the VSMC derived from patients who have developed stenoses are resistant to heparin and different individuals' VSMC display consistent but diverse responses to heparin, will the responses of an individual's VSMC predict the fate of their graft ?

A human cell culture system has been used to further investigate the biology of MIH. Firstly, to investigate if this heterogeneity of responses was confined to patients with known arterial disease. Secondly, a prospective study has been performed to see if there was an association between VSMC resistance to

heparin and the subsequent development of stenoses in the grafts from which these cells were derived.

PATIENTS, MATERIALS AND METHODS

NON-ARTERIOPATHIC PATIENTS

This group comprised 25 patients with sapheno-femoral incompetence suitable for day care surgery being operated on between October 1994 and October 1995. There were 5 smokers (20%). However, none of the rest of this group had recognised risk factors for atherosclerosis.

ARTERIOPATHIC PATIENTS

Between June 1991 and October 1994 eighty four patients undergoing infra-inguinal vein bypass each had a section of redundant vein graft removed at the time of initial surgery and were entered into a standardised graft surveillance program (Cheshire and Wolfe 1993). An angiogram was performed on completion of surgery, followed by scanning of the graft with duplex Doppler ultrasound at 6 weeks, 3 months, 6 months, 9 months and 1 year. Any graft deemed to possess a stenosis detected by ultrasound (in our unit: sensitivity and specificity 100% and 98% respectively) (Taylor et al. 1990) was examined

angiographically for confirmation. All unstenosed grafts were examined angiographically at 1 year. For the purposes of our analysis graft stenosis was defined as a lesion developing between 6 weeks and one year post-operatively, with a V_2/V_1 ratio >2 on duplex Doppler and a reduction in luminal diameter of $>50\%$ on angiography. All angiograms were independently reviewed at a joint surgical and radiological conference without prior knowledge of the results of the heparin inhibition assays.

In the group of patients with known arterial disease the presenting features were: life style limiting (severe) intermittent claudication (8%); rest pain (54%); ulceration (22%) and gangrene (16%). The risk factors for atherosclerosis were: smoking (76%); diabetes mellitus (35%); hypertension (31%); hypercholesterolaemia and hyperlipidaemia (12%).

In situ (ISV) grafting was used in 26 patients and the reversed method (REV) was used in 37 grafts. The site of proximal and distal anastomoses is shown in table 1.

TISSUE CULTURE

Redundant sections of saphenous or cephalic vein were taken from 76 patients at the time of initial infra-inguinal bypass or varicose vein surgery in accordance with local Ethics Committee guidelines, and transported to the laboratory in Hank's balanced salt solution containing 25mM HEPES, penicillin

(200 U/ml), streptomycin (200µg/ml), kanamycin (100µg/ml) and amphotericin B (1.25µg/ml). Vein segments were explanted for VSMC culture within 24 hours of operation. The veins used in this study were all macroscopically normal. No histological examination was made as all tissue was used for cell culture.

VSMC cultures were prepared as previously described (Chapter 3). Veins were carefully dissected to remove the outer adventitia and the endothelium was scraped away with a blade. The remaining inner media containing almost exclusively VSMC (Woolf 1982) was cut into 1mm squares. The resulting explants were placed in 80cm² tissue culture flasks in 5ml of nutrient medium comprising Dulbecco's modified Eagle's medium (DMEM) buffered with 25mM HEPES supplemented with 15% (v/v) foetal calf serum (FCS), l-alanyl-l-glutamine (4mM), penicillin (100U/ml), streptomycin (100µg/ml) and gentamicin (25µg/ml).

Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ (v/v) in air. Over a period of 2 to 6 weeks VSMC migrated from the explants and proliferated to form a confluent monolayer. At this point they were subcultured by trypsinisation into a fresh flask. VSMC were then further cultured and identified by their typical "hill and valley" (Fig 1) morphology and indirect immunofluorescence studies with an antibody to α -actin (Fig 2) which is a marker for smooth muscle cells (Skalli et al. 1987).

CELL PROLIFERATION ASSAY

In order to assay the inhibitory activity of heparin, VSMC at second passage were trypsinised and seeded as previously described at a density of 10^4 cells/ml, 1ml/well in 24 well plates and allowed to attach overnight in standard medium containing 15% FCS. This medium was removed and the cells washed twice with phosphate-buffered saline (PBS) (without calcium and magnesium). The cells were maintained in medium supplemented with 0.4% FCS for 72 hours to growth arrest the cells in the G_0 phase of the cell cycle. Growth stimulation was then commenced with growth medium containing 15% FCS $\pm$ $7\mu\text{M}$ heparin ($100\mu\text{g/mL}$). Triplicate wells were harvested by trypsinisation and counted in an electronic particle counter (Coulter, Luton, UK) on days: 0, 3, 7, 10 and 14 after growth arrest. The growth medium in the remaining wells was replenished at the same time points. The net growth of VSMC in 15% FCS containing growth medium $\pm$ $7\mu\text{M}$ heparin was calculated by subtracting the starting cell number (at the time the cells were released from G_0) from the cell number on day 14. The percentage of heparin inhibition was determined by the following relationship:

$$\% \text{ heparin inhibition} = (1 - (\text{net growth in } 15\% \text{ FCS} + \text{heparin} / \text{net growth in } 15\% \text{ FCS})) \times 100.$$

STATISTICAL ANALYSIS

χ^2 analysis was used to compare group risk factors and the Mann Whitney *U* test to compare the median heparin inhibition values for stenosed and non stenosed grafts.

MATERIALS

FCS was obtained from J. Bio (Les Ulis, France) and all other cell culture materials were obtained from GIBCO Life Technologies/BRL. Heparin (Paynes & Byrne, U.K.) was the kind gift of Dr B. Mulloy, National Institute of Biological Standards and Control, South Mimms, England and had been previously extensively characterised for chemical and anticoagulant properties (Johnson and Mulloy 1976), as well as for its *in vitro* antiproliferative effects on human VSMC (Chan et al. 1992).

RESULTS

In the 25 patients undergoing varicose vein surgery 18 (72%) were female with a median age of 43 years (range of 27 to 47). Cell culture data was available on all of them.

Among the patients with known arterial disease, there were eighty six grafts in as many patients undergoing infra-inguinal bypass surgery for the first time, 63% male and with a median age of 71 (range 32- 90). In 22 patients there was insufficient data: 14 patients completed follow up but their cells died in culture due to infection with *Candida albicans* (of this group 4 patients developed graft stenoses (28.3%)); 5 patients grafts failed during the first month following surgery (5.9%), their graft failure being considered to be due to technical error or thrombosis and 3 patients died with running grafts during the first 6 weeks post-operatively (3.6%). This left 62 grafts which were available for analysis with both surveillance and cell culture data.

The population of patients in each outcome group (stenosis or no stenosis) was not significantly different with respect to age, sex, diabetes, smoking habits and lipid profile (Table 2). Thirty nine patients had angiographically confirmed patent grafts after a minimum of 1 year follow-up and 23 had angiographically confirmed stenoses (Fig 3) that had developed between 6 weeks and 1 year following surgery (37%). All of the latter patients underwent revision surgery and in four cases the intimal hyperplastic lesion was removed and examined histologically. The location of these stenoses is shown in (Fig 4) These lesions were all MIH. The stenoses were first detected at the following times: 9 by 3 months; 16 by 6 months; 21 by 9 months and 23 by 1 year (Fig 5).

Examining the population of 25 patients with no known arterial disease has demonstrated a median VSMC inhibition in response to heparin, calculated from growth assays, of 37.2% (range of 3.1- 74.1%) and for all the arteriopathies

(62 patients both with and without stenoses) a median of 35.8 (range of 0-97.5%) ($p > 0.1$: Mann Whitney). (Fig 6)

Amongst these 23 patients with stenoses, the median VSMC inhibition in response to heparin, calculated from growth assays, was 20.9% (range of 0-62.4%). By contrast the median for those 39 patients whose grafts remain unstenosed was significantly higher at 54% (range was 2.4- 97.5%) ($p < 0.001$: Mann Whitney).(Fig 7).

DISCUSSION

Vascular stenosis, or restenosis, has proved an intractable clinical problem despite intensive attempts to devise effective pharmacological prophylaxis. Although the basic features of MIH are well-described, it is unclear why this process, which is essentially a reactive response to vascular injury (Sottiurai et al. 1983b and Clowes 1993) becomes excessive in 25-50% of patients (Taylor et al. 1990) and leads to clinically significant luminal obstruction. Many therapeutic agents have proved highly effective in attenuating MIH following vascular injury in animal models but this has not been predictive of success in clinical trials, which have been consistently negative (Faxon and Currier 1995). Even if one allows for weaknesses in trial design this suggests that such models may not be wholly appropriate, particularly if they involve species as remote from humans as the rat and rabbit. It is also true that remodelling, perhaps

involving the extracellular matrix, has not so far been a therapeutic target (Lafonte et al. 1996).

The incidence of stenoses is slightly higher than that reported in previous studies (which is in the order of 13-25%) (Szilagyi et al. 1973, Grigg et al. 1988, Moody et al. 1992 and Mills et al. 1995), however, the timing is in keeping with those studies (Berkowitz et al. 1989, Grigg et al. 1988 and Taylor et al. 1990). From this study it appears that the distal bypass segment of vein is at slightly greater risk independent of the method of bypass used, *in situ* or reversed. The reason for this however is unclear from this study. Berkowitz et al. (1989) demonstrated this in a much larger series of 521 reversed grafts, but there is no equivalent data to confirm this finding in *in situ* grafts. In fact, Mills et al. (1993) demonstrated that there was a similar incidence of stenoses at proximal and distal locations of the vein graft. Munro et al. (1994) demonstrated that the sensitivity of VSMC to the growth inhibitory effect of heparin was the same from any site in the saphenous vein. Taking these two findings together it is possible that the intrinsic risk for development of stenoses is peculiar to a given individual but this does not dictate the location of the stenosis.

As mentioned above it has been shown that cells from a particular individual behave consistently over several passages in terms of response to growth inhibition by heparin. Furthermore, this consistency is also seen in cells taken from different vessels in the same patient, including those from stenotic lesions themselves (Munro et al. 1994). By contrast, in confirmation of earlier

observations, we have demonstrated that there is wide inter-individual diversity in responses (Chan et al. 1993).

It is known that endogenous glycosaminoglycans similar to heparin, produced by both endothelial cells and VSMC, are involved in growth regulation of these cells. The concentration of heparin used ($100\mu\text{g/mL}$) is higher than normally attained in serum after therapeutic doses but is similar to concentrations of glycosaminoglycans in the extracellular matrix around the VSMC in the vessel wall (Sisto et al. 1989). The effect of heparin on growth inhibition cannot involve its anti-thrombotic effect as the cells are grown in preclotted foetal calf serum. It is unlikely that the reduced heparin sensitivity of VSMC from patients with stenoses could be due to residual effects of drug treatments because the cells had been passaged twice. The variety of growth potentials of VSMC from non-arteriopathic patients implies that these responses antedate any form of vascular injury and therefore represent a risk factor at the cellular level for progressive MIH and hence vein graft stenosis.

These findings reinforce the assumption that differences in cellular response are reproducible and significant. In the present study I have confirmed earlier observations that VSMC derived from patients with stenoses are relatively resistant to growth inhibition by heparin and extended these findings by demonstrating a highly significant correlation between reduced growth inhibition by heparin in cells cultured from the grafted vessel and the development of stenosis in the subsequent year.

Heparin resistance has been shown in this series to be a highly significant predictor for the development of subsequent stenosis. The conventional risk-factors for atherosclerosis have by contrast shown little or no consistent association with clinical outcome. Also there is no difference in heparin sensitivity between arteriopaths and non-arteriopaths.

Obviously, the next step is to consider possible mechanisms to explain this finding.

FIGURE LEGENDS

Figure 1. Photomicrograph of human VSMC in culture displaying the typical “hill and valley” appearance. Areas of high cell density represent “hills” and areas of low cell density represent “valleys”. The cells were photomicrographed at high power (40x magnification) using a phase contrast microscope.

Figure 2. Photomicrograph of human VSMC stained immunocytochemically for α actin. Growth arrested cells were stained with FITC-labelled antibody and photomicrographed at high power (40x magnification) using a fluorescence microscope.

Figure 3a. Digital subtraction angiogram prior to patients discharge showing patent distal graft and anastomosis.

Figure 3b. Angiogram performed 3 months after bypass showing a vein graft stenosis at the distal anastomosis.

Table 1.

Distribution of section of infra-inguinal artery used as location for proximal anastomosis (inflow vessel) and distal anastomosis (outflow vessel) for saphenous vein grafts.

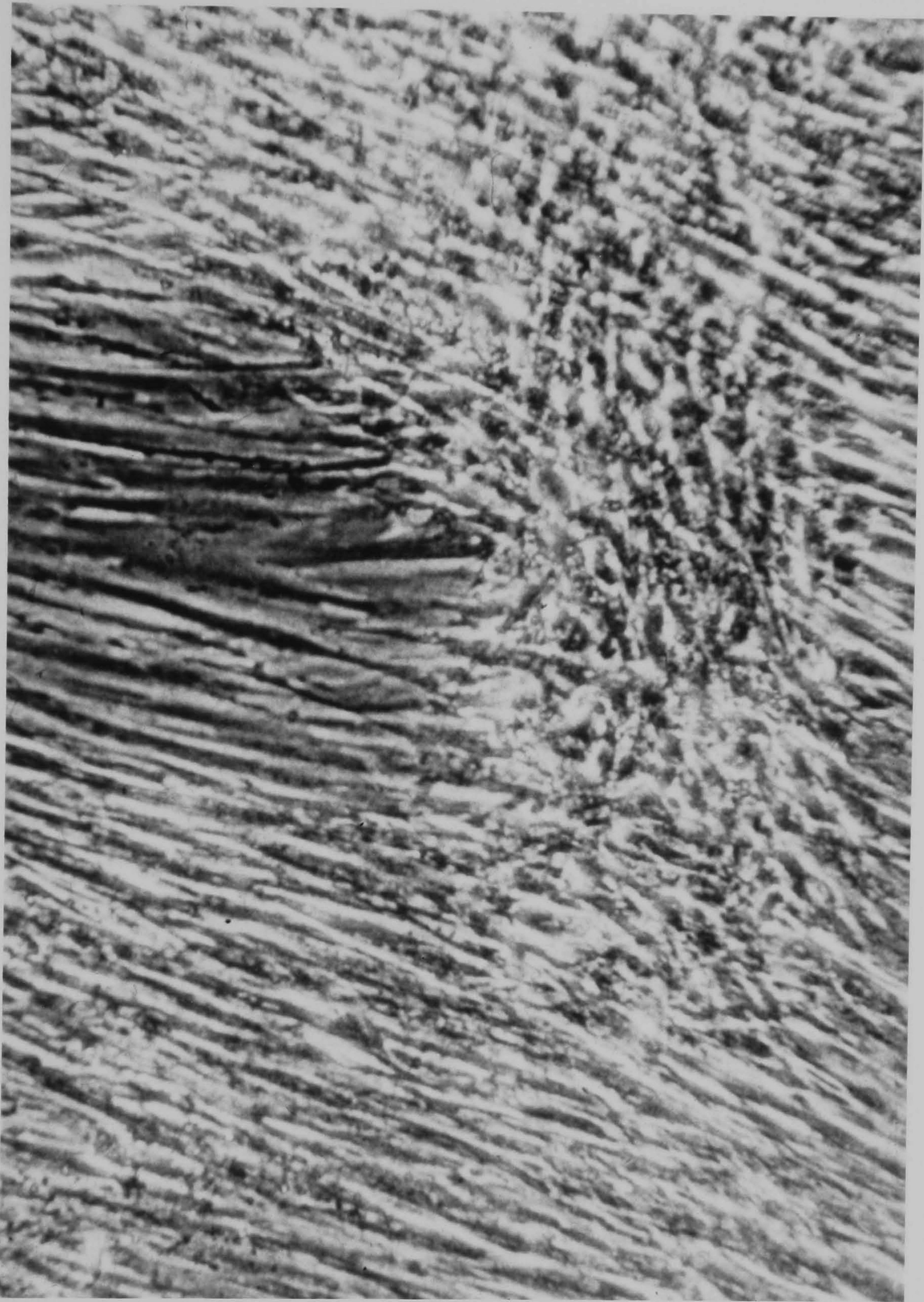
INFLOW VESSEL		OUTFLOW VESSEL	
Common Femoral	58	Above knee popliteal	9
Superficial Femoral	3	Below knee popliteal	25
Popliteal	1	Tibio-peroneal trunk	9
		Anterior tibial	3
		Posterior tibial	11
		Peroneal	5

Table 2

Clinical details of patients included in prospective study.

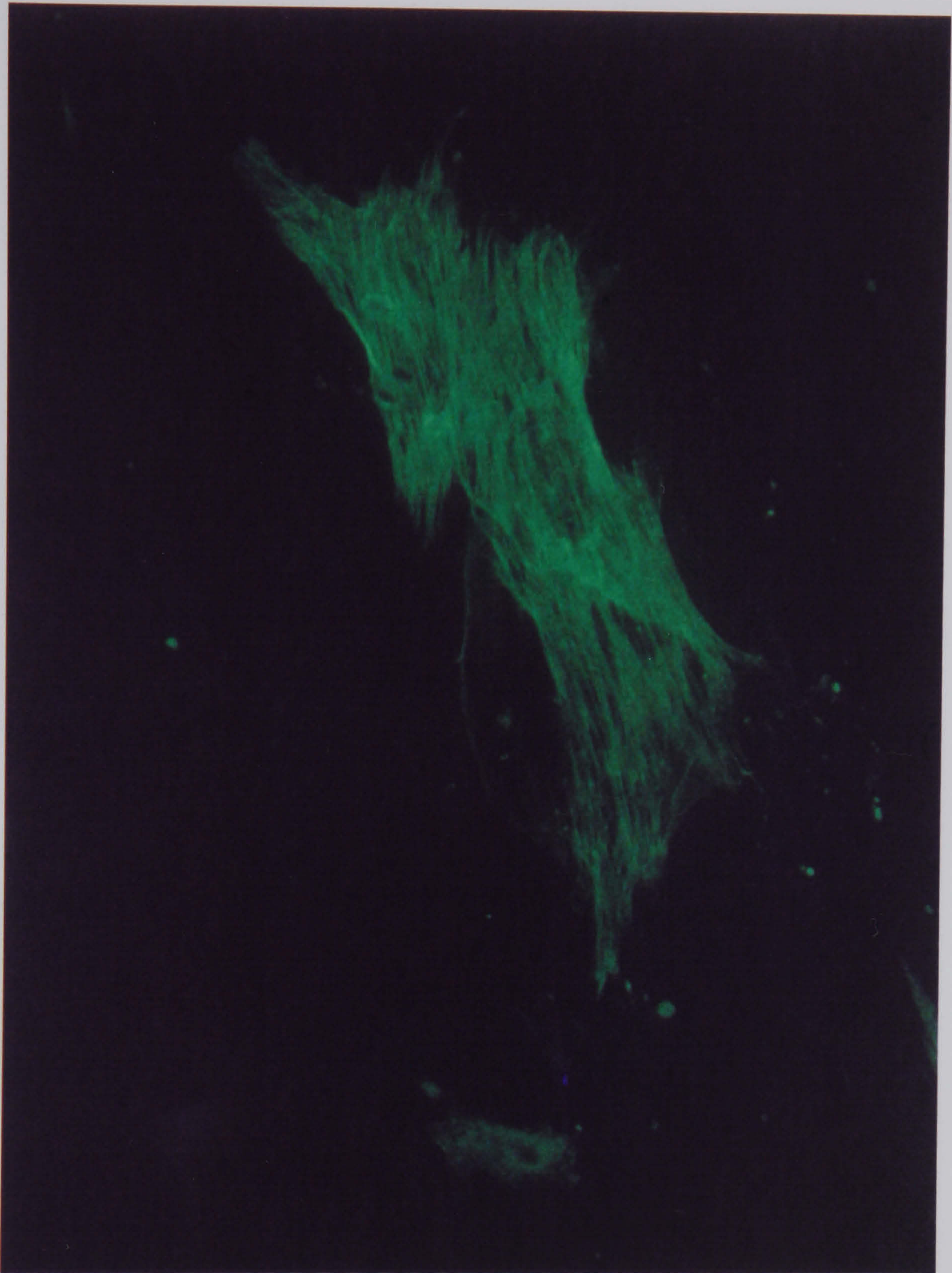
RISK FACTOR	OR	NUMBER OF PATIENTS WITH STENOSIS	NUMBER OR PATIENTS WITH NO STENOSIS
COMORBIDITY			
Male/Female		14/9	28/11
Angina		12	11
MI		7	10
CABG		3	5
Hypertension		9	12
Diabetes		9	13
Hyperlipidaemia		3	5
Smoked pre bypass		18	29
Smoked post bypass		10/18	17/29

Figure 1.



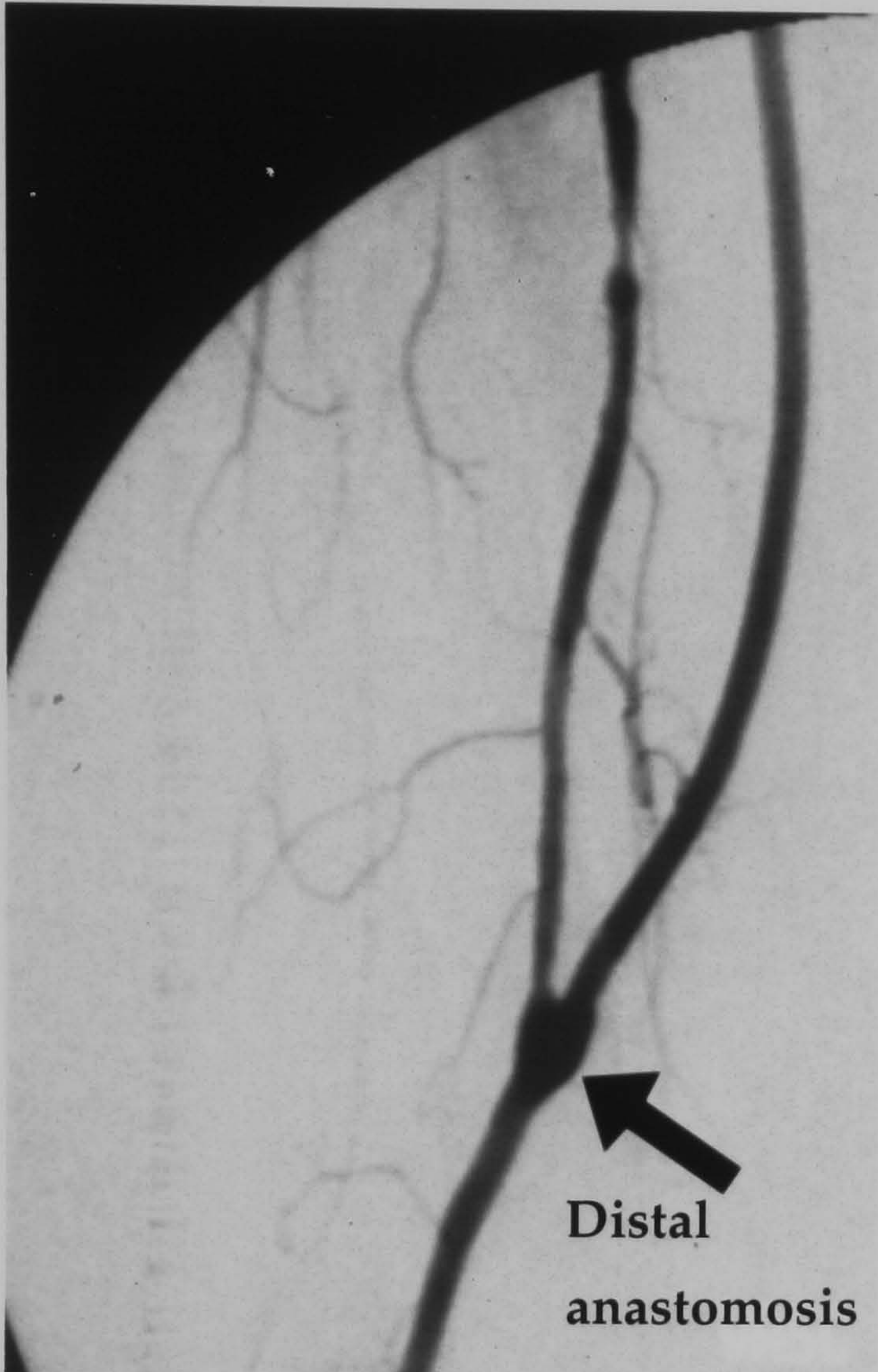
'Hill and valley' appearance of human VSMC in culture.

Figure 2.



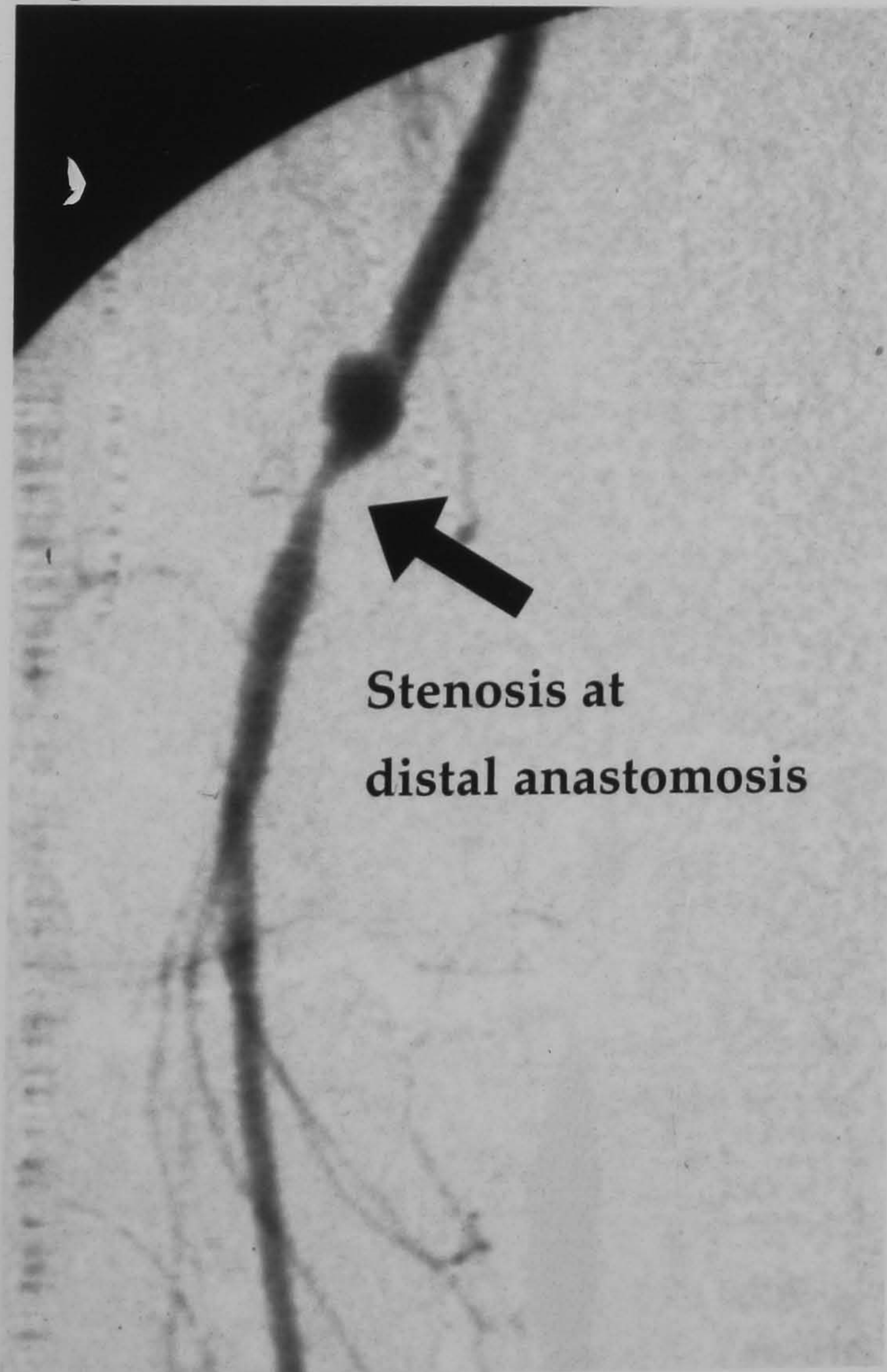
Immuno-cytochemical staining of human VSMC for alpha actin.

Figure 3a.



Completion angiogram showing
patent graft and anastomosis

Figure 3b.



Angiogram at 3 months showing
stenosis at distal anastomosis

Figure 4.

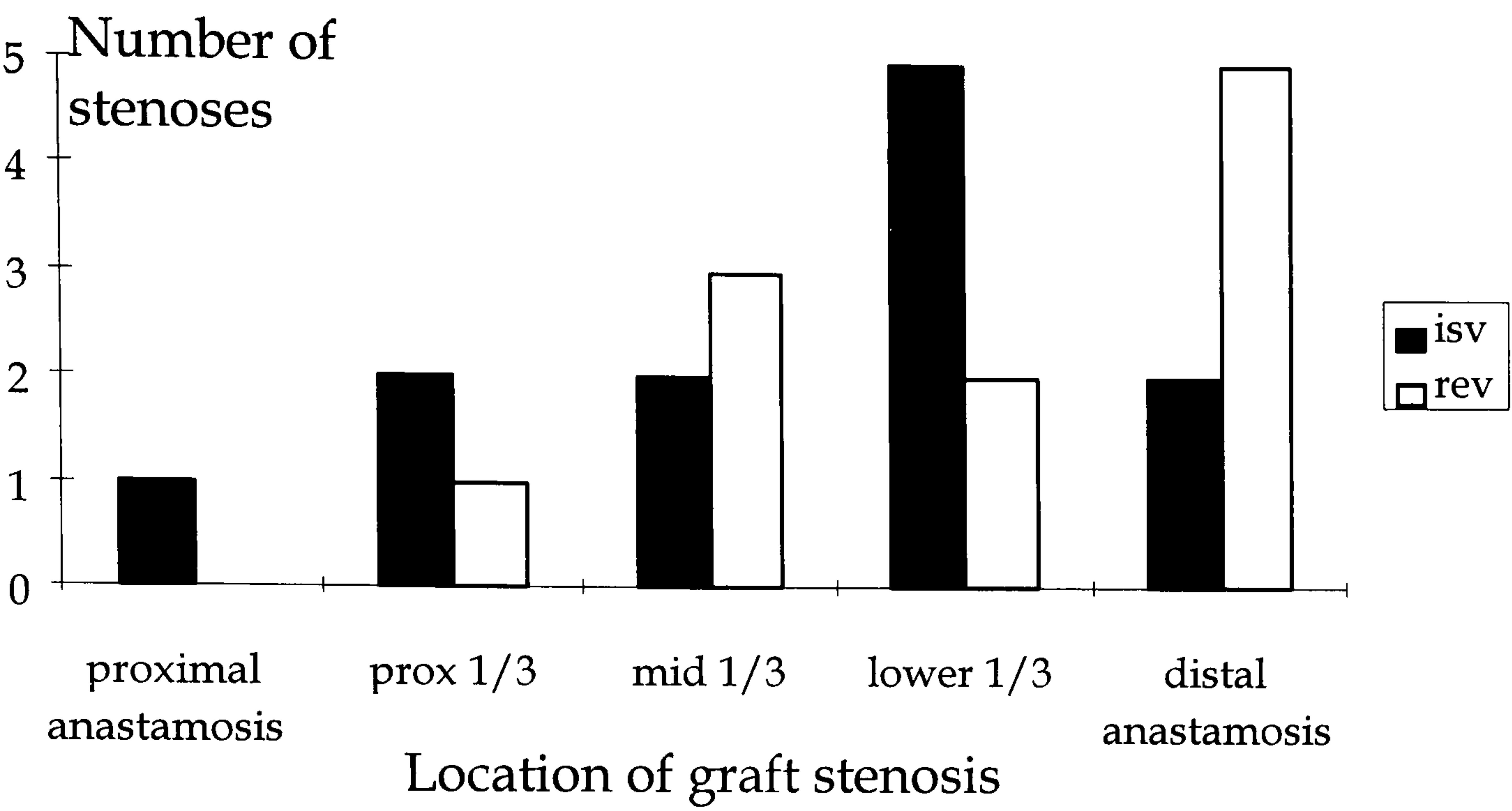


Figure 4. Distribution of stenoses detected in the body of the vein graft and the type of vein graft that these stenoses were found in. In-situ (ISV) or reversed (REV) vein graft.

Figure 5

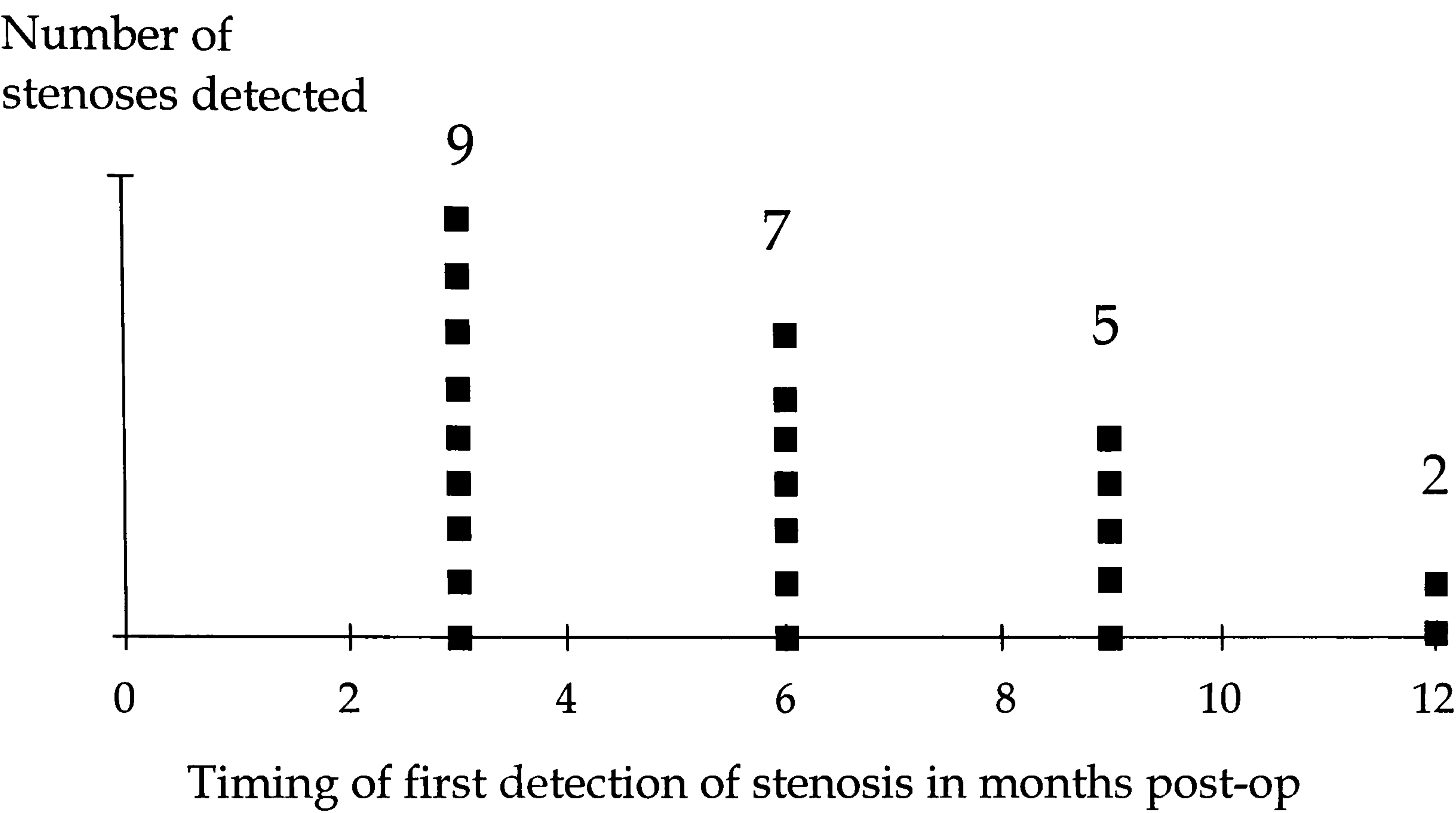


Figure 5. Time points at which the stenoses were detected during the period of vein graft surveillance. Each point represents a stenosis.

Figure 6

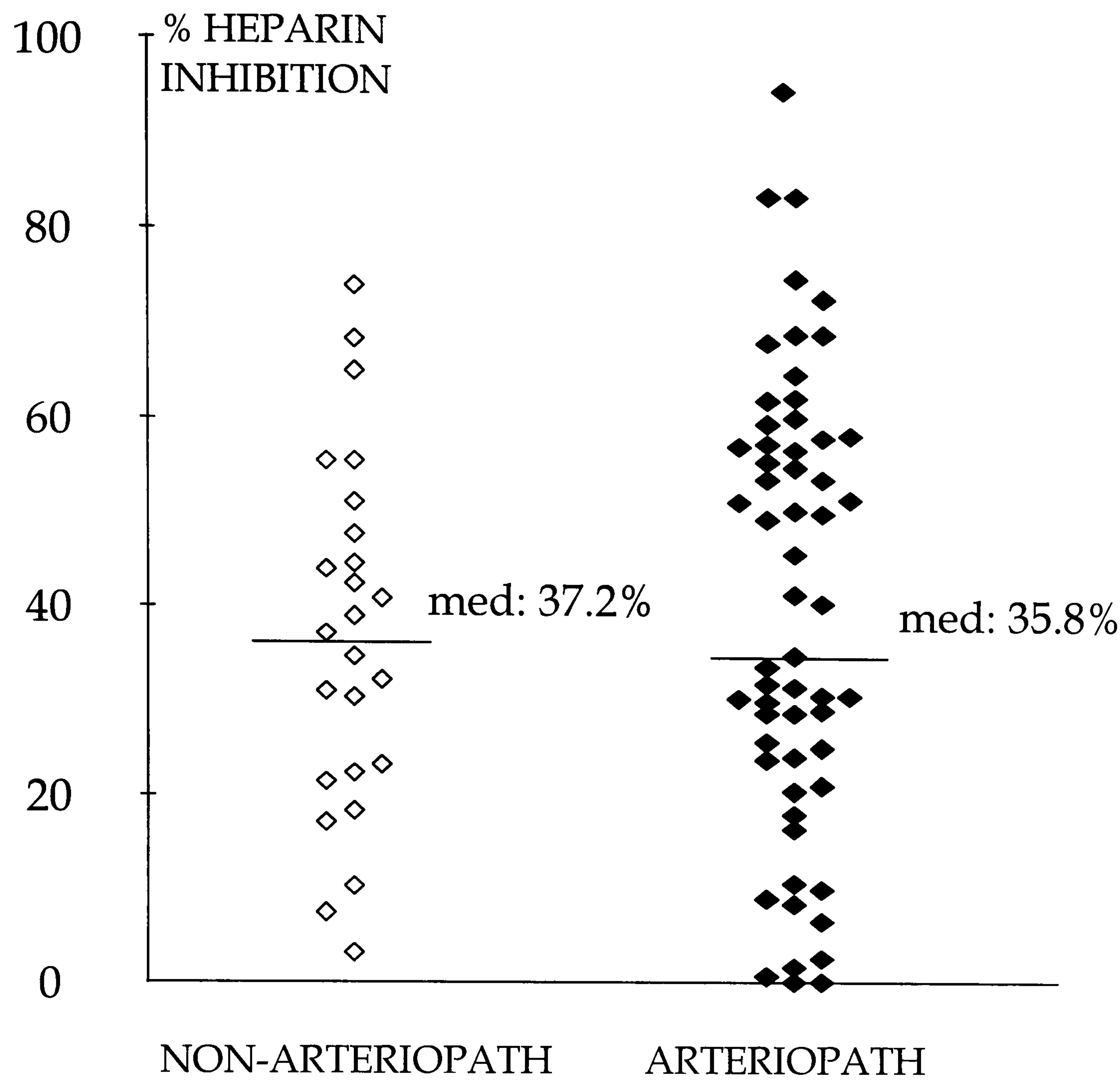


Figure 6. Inhibition of proliferation by heparin (7 μ M) of vascular smooth muscle cells derived from known arteriopathic patients and from those who had no known arterial disease. Inhibition was calculated as described in Methods. Each symbol represents a patient.

Figure 7

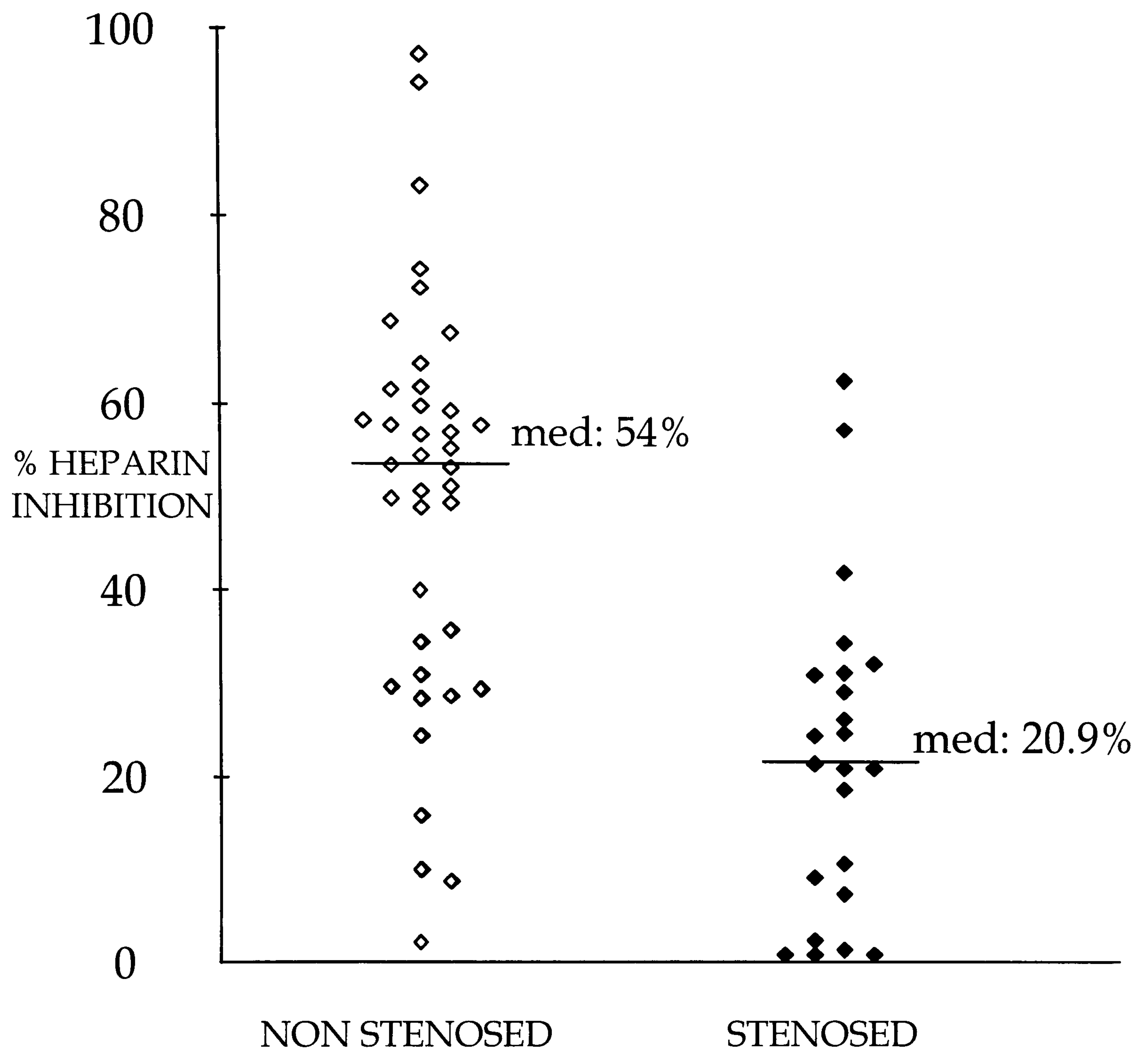


Figure 7. Inhibition of proliferation by heparin ($7\mu\text{M}$) of vascular smooth muscle cells derived from saphenous vein of patients with and without stenosis of infra-inguinal vein grafts between 6 weeks and 1 year of surgery. Inhibition was calculated as described in Methods. Each symbol represents a patient.

HEPARIN BINDING TO HUMAN VSMCs I: KINETICS OF BINDING.

INTRODUCTION

Heparin and heparin like glycosaminoglycans play an important and complex role in the blood vessel wall biology. Anticoagulant activity of heparan sulphate on the endothelial cell surface is crucial in the maintenance of the non-thrombogenic property of blood vessels (Marcum et al. 1986, Marcum and Rosenberg 1989). Heparin augments the mitogenic potency of endothelial cell growth factor and is chemotactic for endothelial cells (Terranova et al. 1985). By contrast, both anticoagulant and non-anticoagulant heparins inhibit VSMC and regulate a variety of VSMC activities. The antiproliferative mechanism by which heparin works is still not clearly understood. Many effects of heparin on growth related events in VSMC have been observed and it is known that heparin acts at multiple points in the growth cycle (see Chapter 3).

Since both endothelial (Castellot et al. 1981) and VSMC (Fritze et al. 1985) secrete heparin like compounds that inhibit growth, these glycosaminoglycans which are normal components of the extra-cellular matrix, are ideal candidates for the regulation of VSMC proliferation in the vessel wall.

Heparin binds to small mammal VSMC and appears to be internalised by receptor mediated endocytosis strongly suggesting the presence of specific heparin receptors on the VSMC surface. The binding of heparin to rat VSMC using ^{125}I and ^3H heparin probes has demonstrated specific saturable binding sites on the VSMC surface with an affinity ($K_d = 10^{-9} \text{ M}$) and number of binding sites (greater than 100,000 per VSMC), this is fairly typical of regulatory molecules with specific surface receptors (Castellot et al. 1985b).

Saphenous vein is the most commonly used conduit for coronary artery and infra-inguinal bypass and therefore is the most appropriate source of VSMC to study, to further our knowledge of MIH in humans. So far there is very little work looking at human VSMC heparin binding and as part of my examination of heparin binding in human VSMC I have performed assays to characterise the binding of heparin to the surface of saphenous vein derived VSMC. This is crucial in performing further experiments, as it will allow me to determine the optimum exposure time to heparin for the human VSMC and what, if any relationship, exists between the affinity of ^3H -heparin binding and the antiproliferative effect of heparin and its structural analogues.

MATERIALS AND METHODS

TISSUE CULTURE

Redundant sections of vein were taken from patients at the time of initial infra-inguinal and coronary artery bypass surgery in accordance with local Ethics Committee guidelines, and transported to the laboratory and VSMC cultured as previously described in Chapter 4.

[³H] HEPARIN BINDING ASSAYS

In order to determine the saturability of heparin binding to human VSMC I examined [³H]-heparin binding to a known heparin sensitive and known heparin resistant cell lines. VSMC were seeded at a density of 10⁵ cells/ml, 1ml/well in 24 well plates and allowed to attach overnight. The standard 15% FCS was replaced with 0.4% FCS (v/v) after two washes with PBS and growth arrested for 72 hours as described above. The cells were then cooled to 0-4°C and washed with ice cold PBS prior to the binding assays which were conducted on ice to prevent internalisation of ligand

SATURATION ASSAY

For saturation binding to be assayed, pre-cooled solutions of [^3H]-heparin from $5 \times 10^{-9}\text{M}$ to 10^{-6}M were prepared in serum free DMEM and added to triplicate wells for 45 minutes and maintained on ice during the assay. Non specific binding was determined by incubation, under the same conditions and at the same time, with pre-cooled solutions of [^3H]-heparin (over the same concentration range) with excess of unlabelled heparin (10^{-5}M). At the end of the incubation period [^3H]-heparin solution was removed and cells were washed three times with ice cold PBS and solubilised in 1M NaOH overnight at room temperature. Cell lysates were transferred to scintillation vials to which Ecoscint (National Diagnostics, Hull, UK) was added. Radioactivity was counted in a liquid scintillation counter (Canberra, Packard, Pangbourne, U.K.) to determine the amount of bound [^3H]-heparin.

TIME COURSE [^3H] HEPARIN BINDING ASSAY.

ASSOCIATION

Time course analysis of binding of radiolabelled heparin was performed on 4 VSMC cell lines. Total binding was assayed by incubation of pre-cooled solutions of [^3H]-heparin (10^{-7}M) prepared in DMEM for up to 120 minutes and maintained on ice during the assay. Non specific binding was assayed by the

addition of pre-cooled solutions of [^3H]-heparin (10^{-7}M) with a 100 fold excess of unlabelled heparin (10^{-5}M) for the same time periods and under the same conditions. Radioactivity was measured as for the saturation assays

DISSOCIATION

Having determined the association time for steady state to be reached, three VSMC lines were incubated, for 30 minutes in 10^{-7}M [^3H]-heparin for total binding and [^3H]-heparin at 10^{-7}M with a 100 fold excess of unlabelled heparin (10^{-5}M) for non specific binding. VSMC were then washed three times in ice cold PBS and 100 fold excess of unlabelled heparin (10^{-5}M) was added. Incubation on ice was maintained for 1, 5, 10, 15, 30, 45, 60, 90 and 120 minutes. At the end of each incubation period, cells were washed again three times with ice cold PBS and solubilised in 1M NaOH overnight at room temperature. Radioactivity was counted as above.

SPECIFICITY OF [^3H]-HEPARIN BINDING

The pharmacology of [^3H]-heparin binding to human VSMC was examined by performing displacement analysis of radioligand binding on 5- 8 cell lines. Assays were conducted on ice for 30 minutes as described above using a range of concentrations (10^{-9}M to 10^{-5}M) of heparin, chondroitin sulphate, dermatan sulphate, a low molecular weight heparin, an highly sulphated heparan,

another less sulphated heparan sulphate and a short chain saccharide. At the end of the incubation period, cells were lysed and radioactivity was counted as above.

CELL PROLIFERATION ASSAY

As described in chapter 4. With heparin and various heparin analogues.

DNA SYNTHESIS ASSAYS

The incorporation of [methyl ^3H]-thymidine was assessed as a measure of de novo DNA synthesis as described previously (Patel et al. 1996) using cells at the third passage (also see chapter 7).

MATERIALS

Stock [^3H]-heparin (1mCi/mg, with an average molecular weight of 14kD) was purchased from Du Pont (Stevenage, U.K.) and [methyl- ^3H]-thymidine (740GBq mmol⁻¹) was purchased from ICN Flow (Thame, U.K). Cell culture plastics, supplements and foetal calf serum (FCS) were obtained from Life Technologies, (Paisley, U.K.). Bovine serum albumin (fraction V) and Ecoscint were purchased from Boehringer Mannheim (Lewes, U.K.) and National Diagnostics (Hull, U.K)

respectively. Trichloroacetic and hydrochloric acids were obtained from Fisher Scientific (Loughborough, U.K.). Unless otherwise stated all other reagents were obtained from Sigma (Poole, U.K.).

Unfractionated porcine heparin (average molecular weight 14000, Payne & Byrne, Birmingham, U.K.), low molecular weight heparin, heparan sulphate fractions and trehalose octasulphate were the generous gift of Dr. B. Mulloy, National Institute of Biological Standards and Control, (South Mimms, U.K.). The unfractionated heparin has previously been characterised for its anticoagulant and chemical properties(Chapter 4). Heparan sulphate fractions I and II have mean molecular masses of 25000 and 8000 daltons respectively (Casu et al. 1983). Heparan sulphate fraction I (HSI) is less sulphated than fraction II (HSII) which in turn is less sulphated than the unfractionated heparin. Low molecular weight heparin (mean molecular mass of 6000) was prepared by fractionation from a bulk sample of heparin and has not been depolymerised either chemically or enzymatically. Trehalose octasulphate is a disaccharide of 1400 daltons.

DATA ANALYSIS

Data are presented as means + s.e.m. of (n) observations. Saturation data from radioligand binding experiments were fitted to a Langmuir function by non-linear regression.

$$Y = \frac{B_{\max} \cdot [\text{Ligand}]}{K_d + [\text{Ligand}]}$$

for one site or

$$Y = \frac{B_{\max 1} \cdot [\text{Ligand}]}{K_{d1} + [\text{Ligand}]} + \frac{B_{\max 2} \cdot [\text{Ligand}]}{K_{d2} + [\text{Ligand}]}$$

for two sites

where Y = specific binding at a given [ligand]. K_{dn} is the dissociation constant and $B_{\max n}$ = maximum number of binding sites.

Displacement data were fitted to a logistic function

$$Y = \frac{100}{1 + 10^{(x + pA_2)}}$$

where Y = specific binding at a [displacer], $x = \log$ [displacer], $pA_2 = -\log IC_{50}$.

IC_{50} values were converted to K_i using the Cheng Prusoff equation (Cheng and Prusoff 1973).

Association data were fitted to a monoexponential function

$$Y = Y_{\max} \cdot (1 - e^{-K_{\text{obs}} \cdot t})$$

where Y = binding at time (t), $Y_{\max}$ = maximum binding, K_{obs} = apparent onset constant. Similarly, the dissociation rate constant was estimated using

$$Y = Y_{\max} \cdot e^{-K_{\text{off}} \cdot t} + c$$

where Y = binding at time (t), $Y_{\max}$ = maximum binding, K_{off} = dissociation rate constant. The true rate constant (K_{on}) was calculated as

$$\frac{K_{\text{on}} = K_{\text{obs}} - K_{\text{off}}}{[\text{Ligand}]}$$

and K_d was estimated as $K_{\text{off}}/K_{\text{on}}$.

RESULTS

Binding of [^3H]-heparin (10^{-7}M) to human VSMC occurred in a concentration-dependent and saturable manner at 0-4 °C as shown in Fig. 1. Non-specific binding of [^3H]-heparin (10^{-7}M) accounted for approximately 15% of the total binding as determined in the presence of 100 fold excess unlabelled heparin (10^{-5}M) under these conditions. In these saturation experiments ($n=2$), data were best fitted by a single site model. The mean K_d derived from these saturation assays was 120nM (range 110 – 129nM, $n = 2$). B_{max} was calculated from these data to represent $2 - 4 \times 10^6$ sites/cell.

[^3H]-heparin (10^{-7}M) binding to human VSMC reached steady state conditions within 30 minutes at 0- 4 °C (Fig. 2a) and the apparent onset rate constant (K_{obs}) was $0.376 \pm 0.100\text{M}^{-1}\text{s}^{-1}$ ($n=4$). Dissociation experiments (Fig. 2b) showed that about 65% of the bound [^3H]-heparin was displaced by the addition of a 100 fold excess cold heparin (10^{-5}M) and that the offset rate constant was $0.087 \pm 0.038 \text{ M}^{-1}\text{s}^{-1}$ ($n=3$). The calculated onset rate constant was calculated to be 2890083 (M^{-1}). The $-\log K_d$ for [^3H]-heparin binding was determined as -7.5 ± 0.4 ($K_d = 30\text{nM}$) (11- 83nM geometric standard error, $n=2$) which compares with the K_d value of 120nM calculated from the saturation data. Neither the association nor the dissociation data were better fitted using a double exponential model, hence providing no evidence of two classes of [^3H]-heparin binding sites from the kinetic studies.

The pharmacology of [^3H]-heparin binding was assessed by examining the displacement of [^3H]-heparin binding by unlabelled heparin or structurally analogous glycosaminoglycans (over the concentration range 10^{-9} to 10^{-5} M) as shown in Fig. 3. Unfractionated heparin, as well as most of the structural analogues competitively inhibited the binding of [^3H]-heparin to human VSMC. The K_i values for heparin and the structural analogues (Table 1) were determined by the Cheng Prusoff equation using 30nM as an estimate of the affinity of [^3H]-heparin for its binding site. The rank order of affinity for the heparin binding site showed that the unfractionated heparin had the greatest affinity, while the highly sulphated sugar trehalose octasulphate was virtually ineffective at displacing [^3H]-heparin from its binding site. With regard to the other glycosaminoglycans, the K_i values showed that [^3H]- heparin binding was not significantly affected until the concentration of the structural analogues was almost 20 fold greater than that of the ligand.

The effect of unfractionated heparin and structural analogues on human VSMC was assessed using DNA synthesis and proliferation assays. In the proliferation assays which were conducted over 14 days, VSMC cultures displayed a range in the sensitivity to the antiproliferative effect of heparin in agreement with my previous findings(Chapter 4)and those of Chan et al. (1993). There was a good correlation between the degree of inhibition of FCS-stimulated growth by heparin ($100\mu\text{g ml}^{-1}$) at 14 days and its effect on DNA synthesis either at the same concentration (Spearman coefficient $r = 0.61$, 95% confidence interval 0.32 – 0.80, $p < 0.0002$) as shown in Fig. 4 or at 1mg ml^{-1} (Spearman coefficient $r = 0.52$, 95% confidence interval 0.20 – 0.74, $p < 0.005$, Personal communication

from M Patel, data not shown). The effect of heparin and some of the other structural analogues was examined on [³H]-thymidine incorporation in these human saphenous vein-derived VSMC for comparison with the pharmacology determined in the radioligand binding studies. The rank order of potency with regard to the inhibition of DNA synthesis was as follows: heparin > heparan sulphate II > low molecular weight heparin >> trehalose octasulphate (Fig. 5) (Patel et al. 1999). I have also examined the effect of heparin (100µg ml⁻¹), both heparan sulphate fractions, low molecular weight heparin and trehalose octasulphate on the proliferation of human VSMC over 14 days using a single (molar equivalent) concentration of the analogues and observed that the inhibition of growth is in keeping with the DNA synthesis experiments (personal communication from M Patel, data not shown). Chelation of calcium ions has been shown not to have any effect on the level of [³H]-heparin binding under these conditions, indicating that [³H]-heparin binding to cell surface sites in these cells was independent of Ca²⁺ (Patel et al. 1999).

DISCUSSION

In this investigation I have characterised the binding of [³H]-heparin to human VSMC grown from explant cultures of human saphenous vein and compared the pharmacology of binding with the ability of structural analogues to inhibit DNA synthesis and VSMC proliferation. Ligand binding studies revealed that [³H]-heparin bound to human VSMC in a specific, time and concentration-dependent manner to a single class of heparin binding sites. The ligand-

receptor interaction was both saturable and reversible indicating that it appeared to occur on the cell surface presumably either to an integral cell membrane protein or an extracellular matrix protein tightly associated with the cell surface.

The estimated K_d for [^3H]-heparin binding from saturation and kinetic studies ranged between 30 – 120nM. A similar affinity (31nM) was estimated for unlabelled unfractionated heparin from displacement studies. Previous reports have defined specific high affinity, protease sensitive binding sites in rat aortic VSMC (approximately 10^5 sites/cell, K_d of 1nM) (Castellot et al. 1985b, Resink et al. 1989 and Letourneur et al. 1995ab) whereas one isolated study with human monocytes identified 1.9×10^6 heparin binding sites/cell with a K_d of 190nM (Leung et al. 1989). Given the differences in B_{max} and K_d in particular, it cannot be assumed that these represent the same site as characterised in this study.

Heparin has been reported to bind to VSMC and subsequently undergo internalisation at 37°C (Castellot et al. 1985b, Sing et al. 1993). Once internalised, it has been localised to endocytic vesicles close to the nucleus and undergoes rapid degradation before ultimately being cleared from cells (Castellot et al. 1985b; Barzu et al. 1987). There is some controversy regarding the entry of heparin into the nucleus. For example, there are reports of the nuclear accumulation of heparin fragments (Busch et al. 1992) and fluoresceinamine-labelled heparin (Sing et al., 1993) while more recent reports have failed either to detect heparin in the nucleus using confocal microscopy (Barzu et al. 1996) or an action on the AP-1 transcription factor (Watanabe et al. 1993; Au et al. 1994).

Comparison of the affinities for heparin, heparan sulphate fraction II, low molecular weight heparin and trehalose octasulphate in the ligand binding studies with their potencies in preventing VSMC proliferation shows a strong correlation however there are marked differences with unfractionated and low molecular weight heparin being several hundred fold more potent in binding than DNA synthesis assays (Patel et al. 1999). Although the binding assays were conducted at 4°C and the DNA synthesis assays at 37°C, it seems unlikely that this difference could account for such marked discrepancies in potency, particularly as binding affinity is generally increased at higher temperature. Similarly, the rank order of potency shows differences between the two assays most notably for the low molecular weight heparin and heparan sulphate II fractions. At 37°C, the binding of heparin is followed by internalisation (Castellot et al. 1985b) and its subsequent degradation (Letourneur et al. 1995ab). The disparity between the affinity of heparin and its inhibitory effect on DNA synthesis has also been reported by Letourneur et al. (1995a) and may be due to differences either in the internalisation and/or metabolism of the glycosaminoglycan which have not been examined in this study.

In summary, the binding of [³H]-heparin to human VSMC occurs through a single class of sites in a specific, saturable, reversible, time and concentration-dependent manner. The rank order of potency for heparin and structural analogues in [³H]-heparin binding studies deviates from their actions on DNA synthesis. This data appears to argue against a simple relationship between cell-associated heparin/anologue binding and the inhibition of DNA synthesis and

suggests that internalisation and/or metabolism of glycosaminoglycans may also determine their inhibitory action on DNA synthesis.

In the next chapter I shall address the issue of the variability in the number of binding sites on the surface of the VSMC and relate this to the heterogeneity of responses observed when the antiproliferative agent is added to exponentially growing VSMC.

Table 1 Comparison of the potencies of unfractionated heparin and its structural analogues in radioligand heparin binding and DNA synthesis assays.

<i>Glycosaminoglycan</i>	<i>[³H]-heparin binding</i> <i>K_i (M)</i>	<i>DNA synthesis</i> <i>IC₅₀ (M)</i>
Heparin (unfractionated)	2.46 × 10 ⁻⁸ (2.41 – 2.51 × 10 ⁻⁸)	2.30 × 10 ⁻⁵ (1.21 – 4.36 × 10 ⁻⁵)
Low molecular weight heparin	5.91 × 10 ⁻⁷ (5.72 – 6.11 × 10 ⁻⁷)	2.01 × 10 ⁻⁴ (4.17 × 10 ⁻⁶ – 9.70 × 10 ⁻³)
Dermatan sulphate	1.33 × 10 ⁻⁶ (1.29 – 1.38 × 10 ⁻⁶)	ND
Chondroitin sulphate	2.39 × 10 ⁻⁶ (2.36 – 2.42 × 10 ⁻⁶)	ND
Heparan sulphate fraction II	3.32 × 10 ⁻⁶ (3.14 – 3.50 × 10 ⁻⁶)	1.01 × 10 ⁻⁴ (1.28 × 10 ⁻⁵ – 7.98 × 10 ⁻⁴)
Heparan sulphate fraction I	8.78 × 10 ⁻⁷ (7.60 × 10 ⁻⁷ – 1.01 × 10 ⁻⁶)	ND
Trehalose octasulphate	>10 ⁻⁵	> 10 ⁻³

FIGURES AND LEGENDS

Legend for Table 1. IC₅₀ values were estimated using non linear regression analysis of the data derived from DNA synthesis experiments on 8 different VSMC strains using heparin at 100mg ml⁻¹. K_i values were calculated by the Cheng Prusoff equation using 30nM as the estimate of the affinity of [³H]-heparin binding for the heparin binding site from experiments on 5-8 different cell strains. ND= not determined. 95% confidence intervals are expressed in parantheses.

Figure 1.

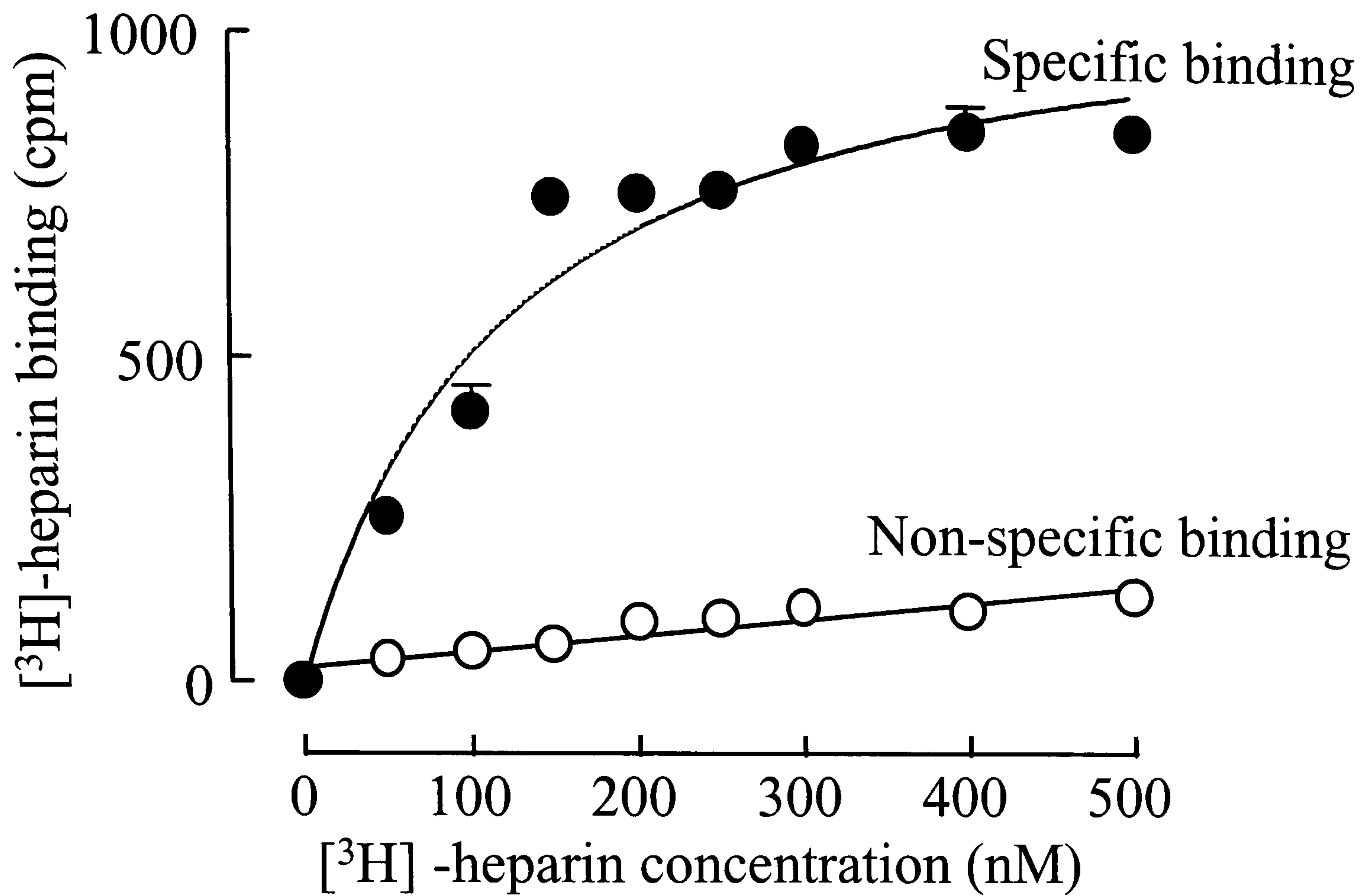


Figure 1. Saturation isotherm of [³H]- heparin binding to human vascular smooth muscle cells. Radioligand binding was performed on ice for 45 minutes with [³H]- heparin (10⁻⁷M) either in the presence or absence of 100 fold excess unlabelled heparin. This figure is a representative isotherm of two experiments performed in triplicate on different human cell strains with each point showing the mean $\pm$ s.e.mean at each time point. Non-specific binding was subtracted from total binding to obtain specific binding. The K_d and B_{max} were calculated as 110nM and 952cpm (i.e. $\sim 4 \times 10^6$ sites cell⁻¹) in the above experiment.

Figure 2a.

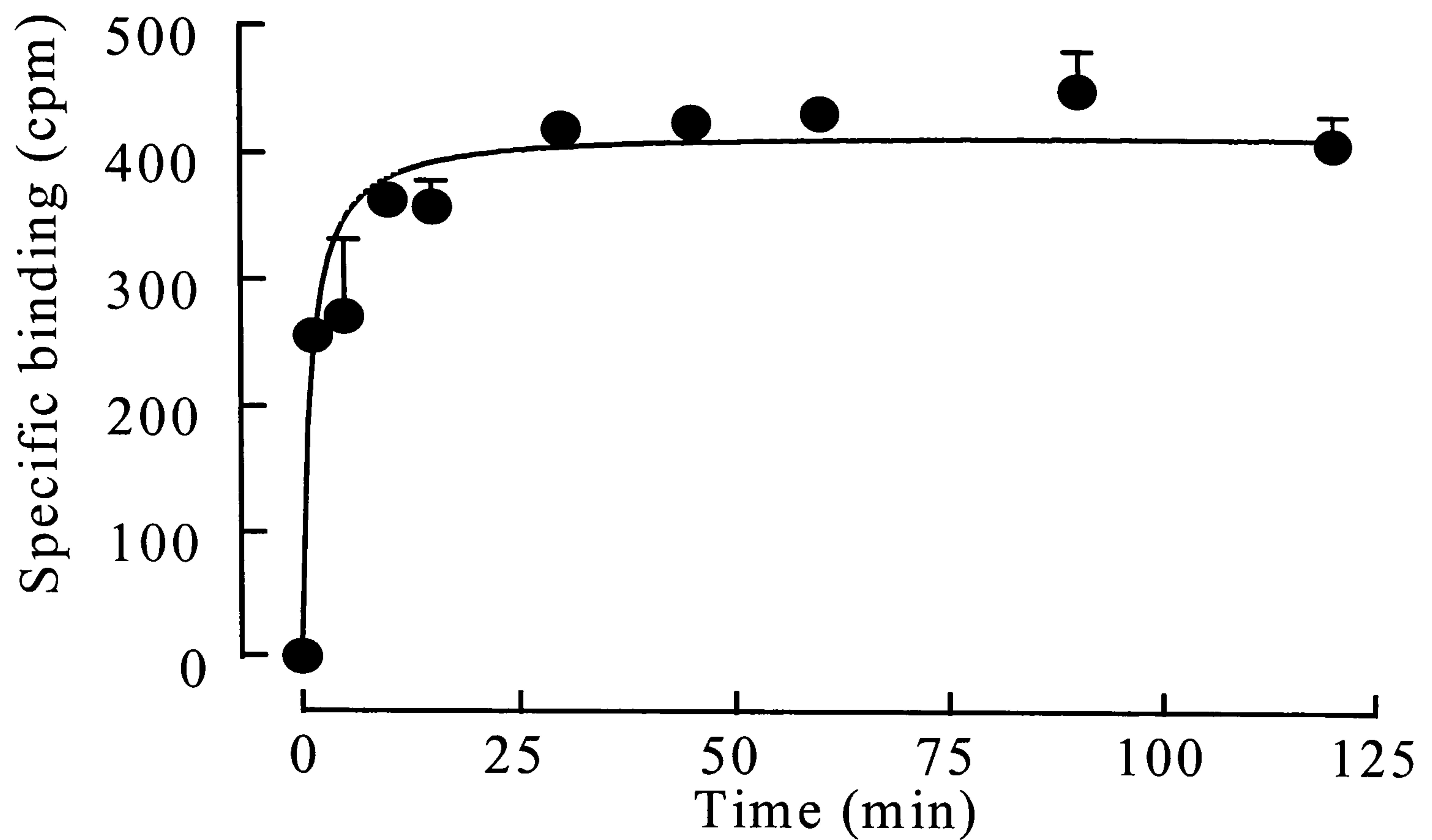


Figure 2a. Onset of $[^3\text{H}]$ -heparin binding to human vascular smooth muscle cells. $[^3\text{H}]$ - heparin (10^{-7}M) binding was performed for 120 minutes in the presence or absence of 100 fold excess unlabelled heparin. This figure shows a representative example from four experiments performed on different cell strains with each point showing the mean $\pm$ s.e.mean of triplicates at each time point. Non-specific binding was subtracted from the data which were fitted to a monoexponential association equation as described in the Methods section.

Figure 2b

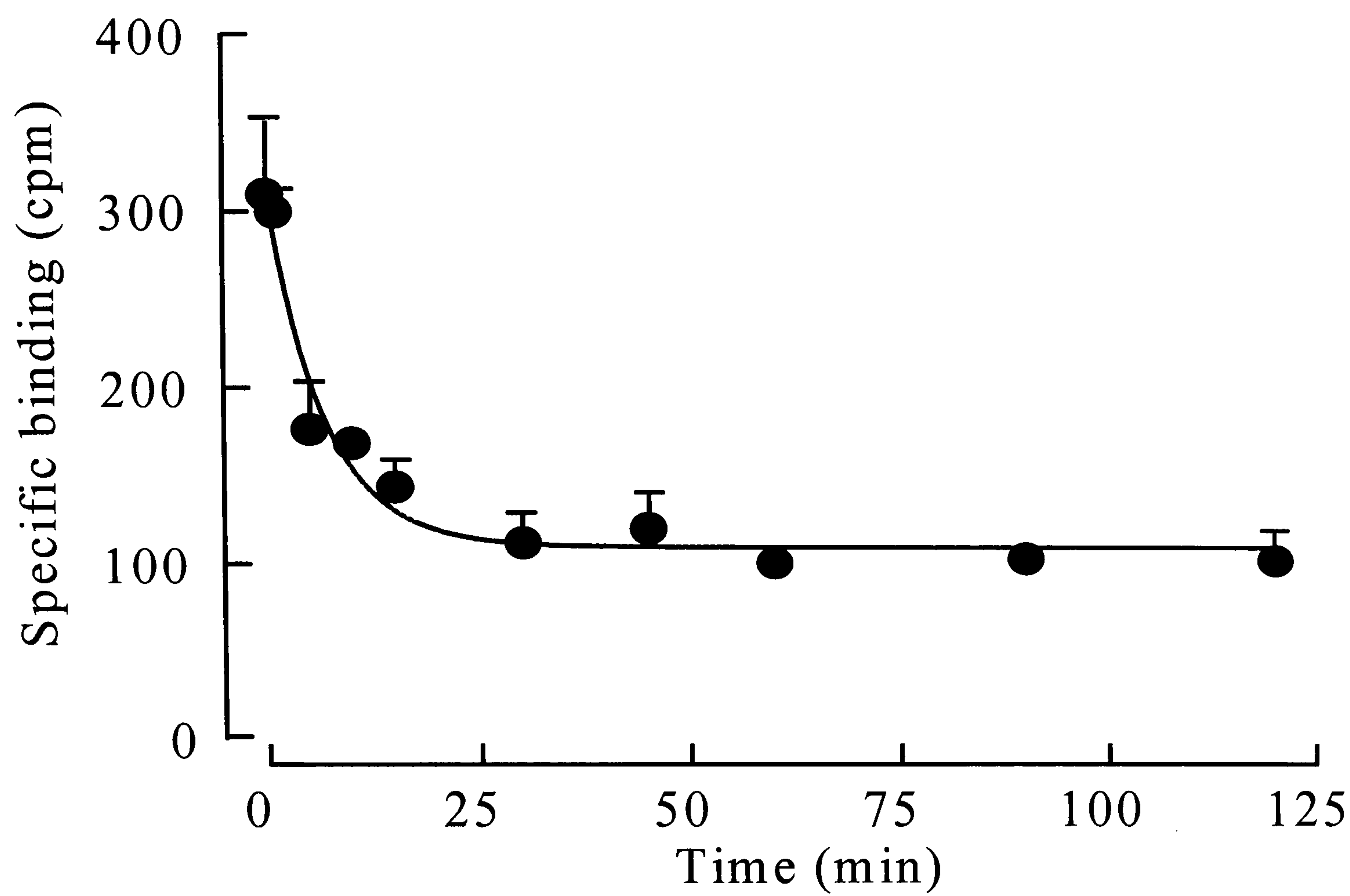


Figure 2b. Offset of [³H]-heparin binding from human vascular smooth muscle cells. Cells were preincubated with [³H]- heparin (10⁻⁷M) for 30 minutes prior to the addition of unlabelled 100 fold excess heparin. Binding was measured at times up to 120 minutes following addition of unlabelled heparin. This figure is representative of three experiments performed on different cell strains with each point showing the mean $\pm$ s.e.mean of triplicates at each time point. The data set are fitted to a monoexponential dissociation equation after subtraction of non specific binding.

Figure 3a

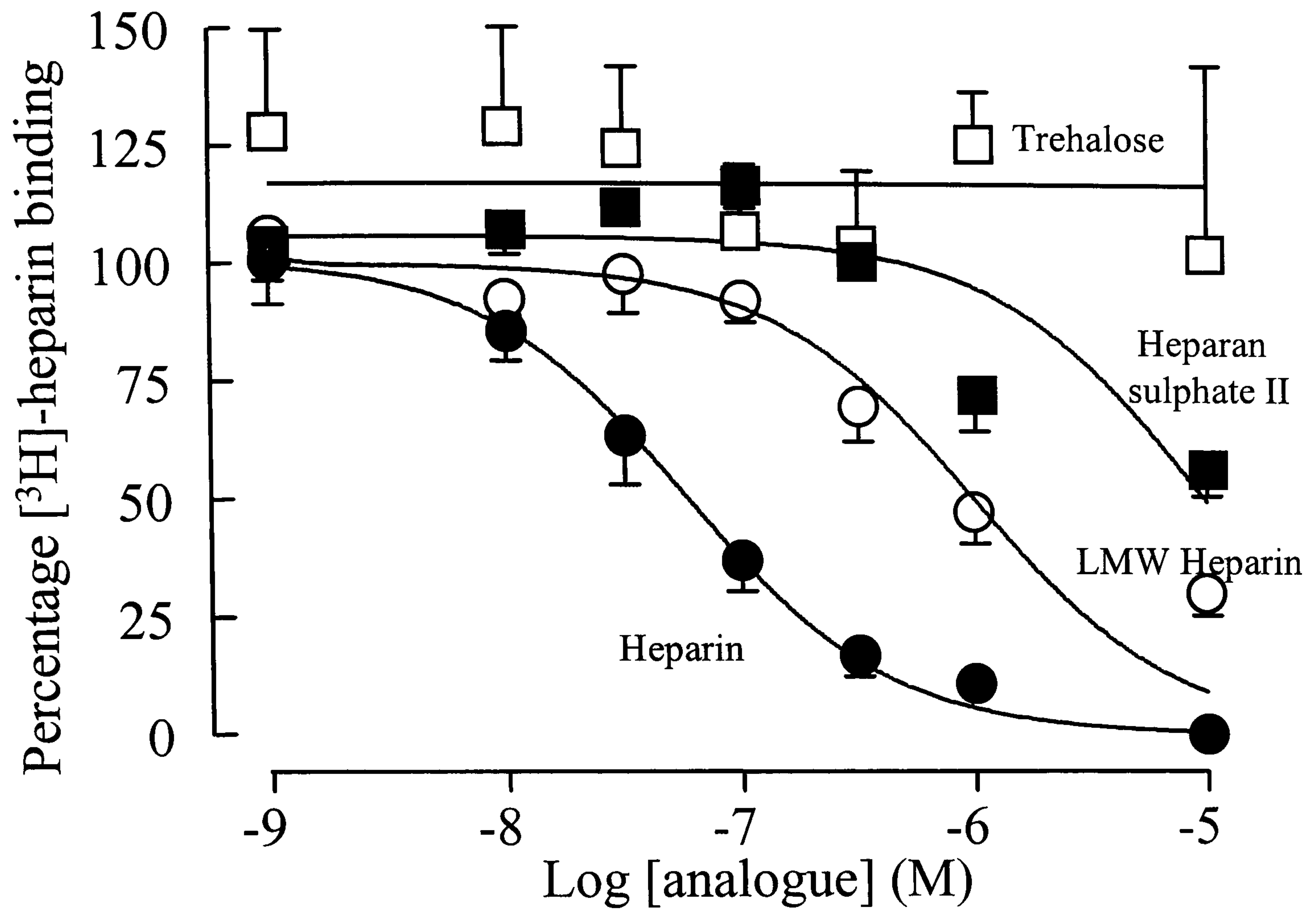


Figure 3a. Pharmacology of $[^3\text{H}]$ - heparin binding to human VSMC. Displacement assays were performed using $[^3\text{H}]$ - heparin (10^{-7}M) in the presence of the above analogues (over a range 10^{-9}M to 10^{-5}M) during the 30 min period of incubation with ligand. Experiments were conducted on 5-8 different VSMC strains.

Figure 3b.

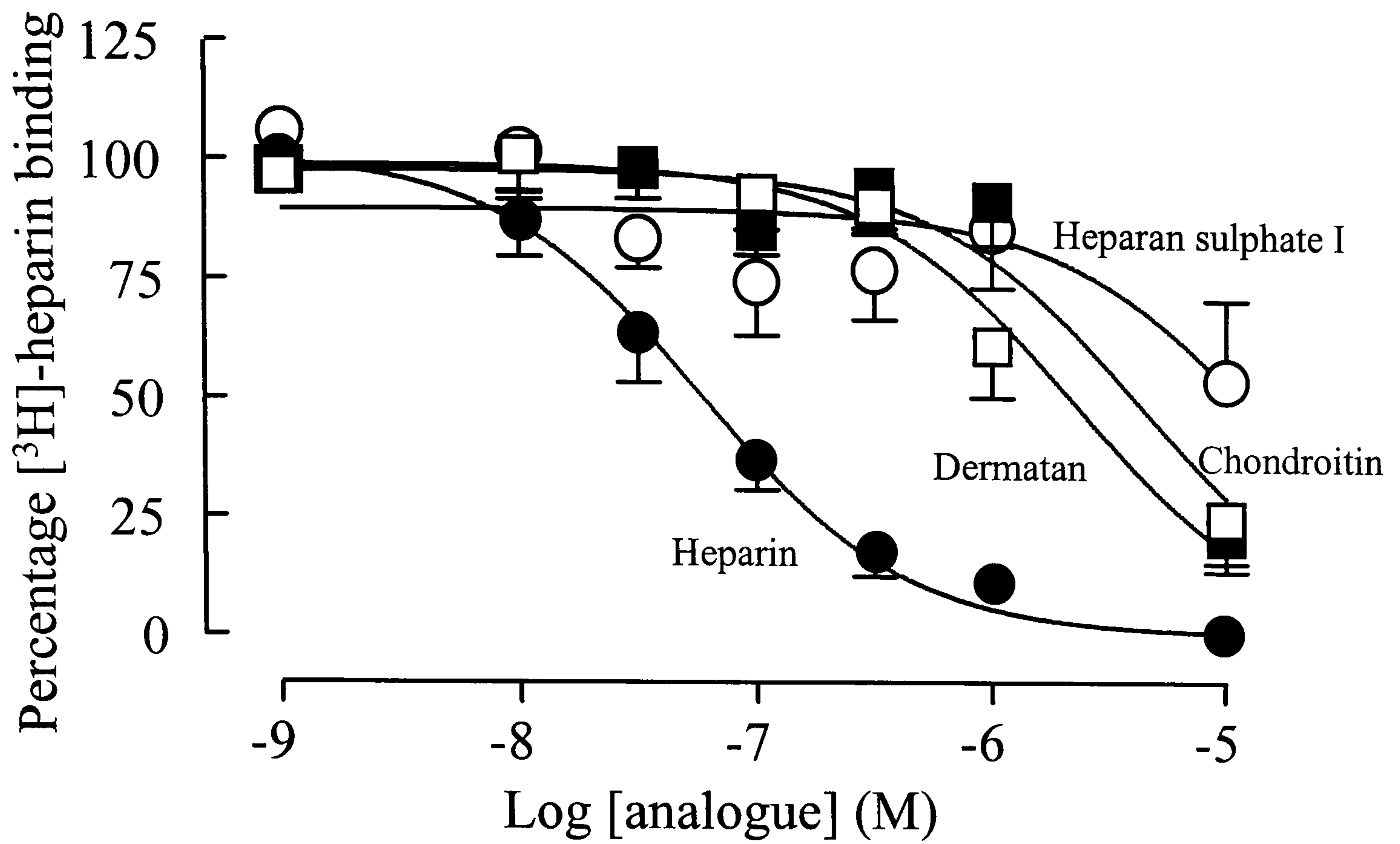


Figure 3b. Pharmacology of $[^3\text{H}]$ - heparin binding to human VSMC. Displacement assays were performed using $[^3\text{H}]$ - heparin (10^{-7}M) in the presence of the above analogues (over a range 10^{-9}M to 10^{-5}M) during the 30 min period of incubation with ligand. Experiments were conducted on 5-8 different VSMC strains.

Figure 4.

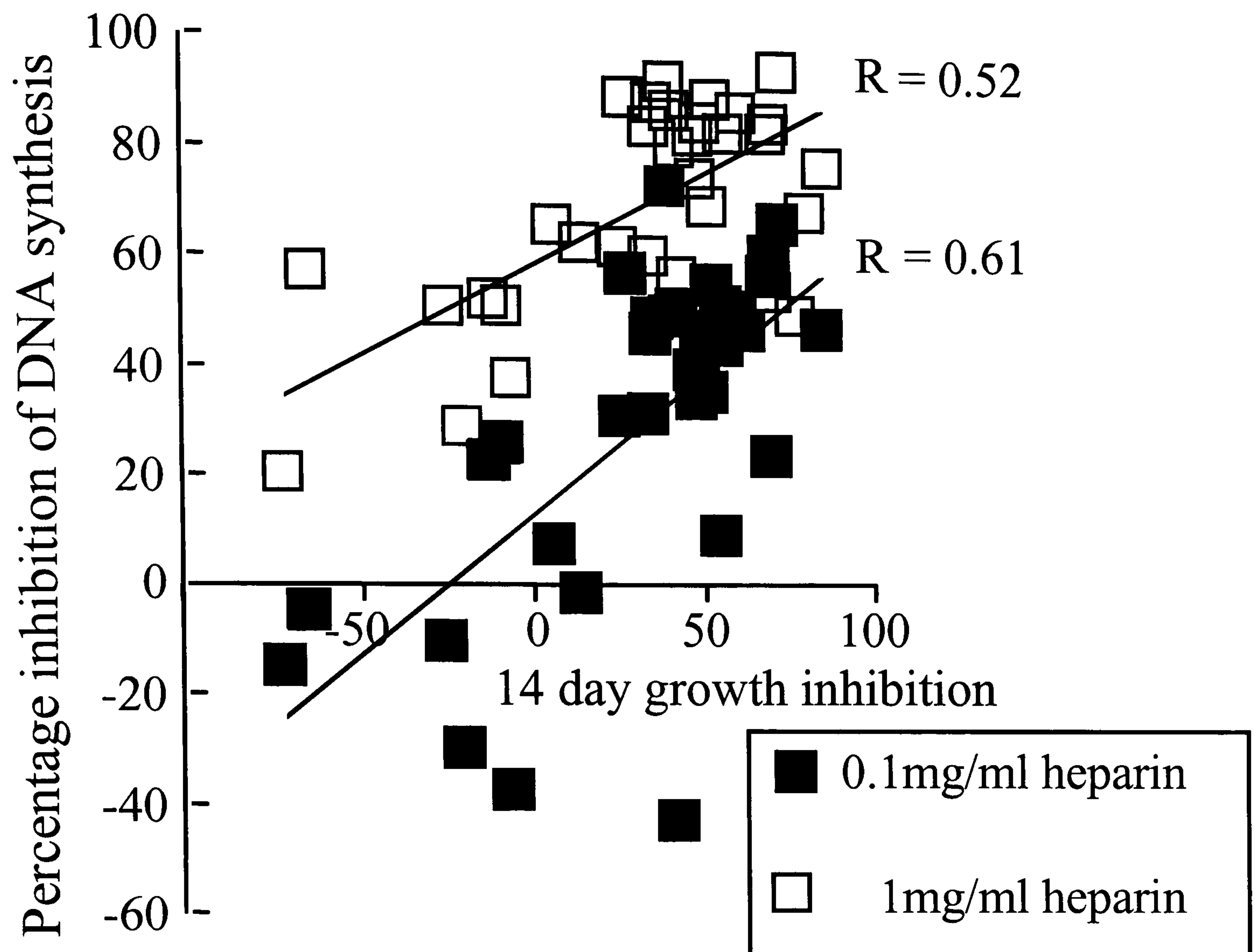


Figure 4. Relationship between the effect of heparin ($100\mu\text{g ml}^{-1}$, $7.14\ \mu\text{mol l}^{-1}$) on human VSMC proliferation over 14 days and DNA synthesis assays. Data points represent the mean ($\pm$ s.e.mean) values obtained (for individual cell strains) from DNA synthesis and proliferation experiments which were conducted simultaneously.

Figure 5.

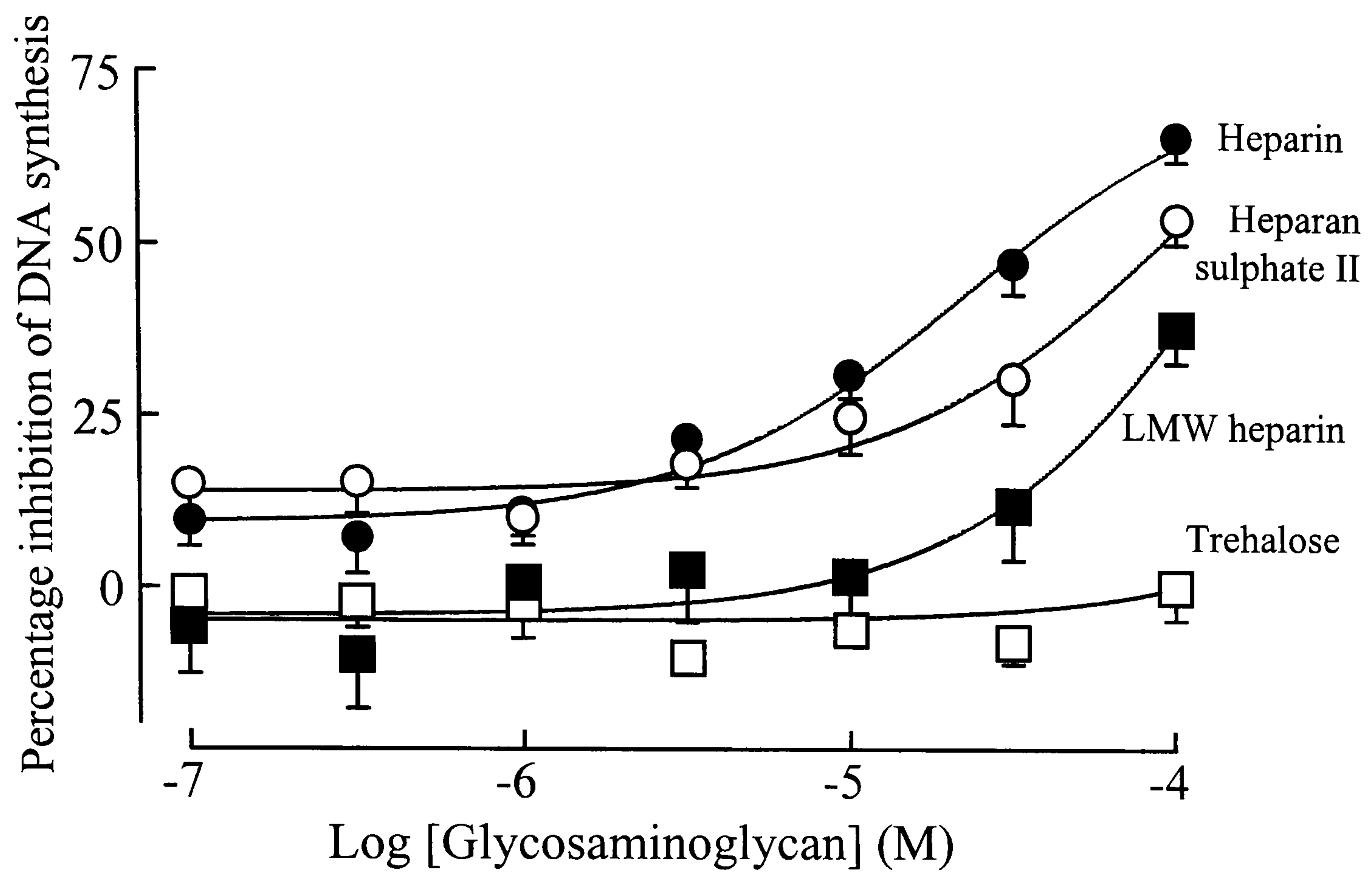


Figure 5. Effect of heparin and structural analogues on DNA synthesis in human saphenous vein-derived VSMC. Experiments were performed in triplicate and data points (mean $\pm$ s.e.mean, $n=8$) represent the percentage inhibition of DNA synthesis with respect to 15% FCS.

HEPARIN BINDING TO HUMAN VSMCs II: CORRELATION BETWEEN BINDING, PROLIFERATION AND CLINICAL OUTCOME.

INTRODUCTION

Heparin was the first relatively specific antiproliferative agent shown to inhibit the growth of VSMC both *in vivo* (Clowes and Karnowsky 1977, Guyton et al. 1980) and *in vitro* (Hoover et al. 1980). It remains unclear how heparin exerts its antiproliferative effect, but similar endogenous glycosaminoglycans, the heparan sulphates, exist in the extra-cellular matrix (Fransson 1987) and are thought to be involved in phenotypic and growth regulation helping to maintain VSMC in a contractile non-proliferative state (Stadler et al. 1989, Campbell and Campbell 1985). This is a property shared by heparin (Chamley-Campbell and Campbell 1981).

It has been shown that VSMC from patients with proven stenoses are relatively resistant to the antiproliferative effect of heparin (Chan et al. 1993) and that VSMC obtained from a variety of patients undergoing vein bypass procedures (cardiac or infra-inguinal) exhibit a very wide range of responses to this antiproliferative action (0-80% inhibition). Amongst this group approximately

30% are likely to develop MIH lesions severe enough to cause clinically significant graft stenoses (Taylor et al. 1990).

In this second study of heparin binding I intend to explore a potential mechanism underlying the variation in heparin responsiveness of human VSMC. This was based on observations which suggested that the binding of heparin to VSMC is required for its growth inhibitory action (Resink et al. 1989, Barzu et al. 1994, Edwards and Wagner 1992). In the spontaneously hypertensive rat (SHR), which demonstrates increased VSMC proliferation and vascular wall hypertrophy, cultured VSMC have fewer heparin binding sites and less sensitivity to growth inhibition by heparin than normotensive WKY controls (Resink et al. 1989). In order to determine whether the mechanism of heparin resistance in human VSMC is related to the extent of heparin binding to the cell surface I have analysed the binding characteristics of radiolabelled heparin in cultured VSMC and compared them with their growth responses to fetal calf serum in the presence and absence of heparin.

MATERIALS AND METHODS

CELL PROLIFERATION ASSAY

Tissue culture and cell proliferation assays were performed as described in chapter 4. The percentage of heparin inhibition was determined by the following relationship:

$$\% \text{ heparin inhibition} = (1 - (\text{net growth in } 15\% \text{ FCS} + \text{heparin} / \text{net growth in } 15\% \text{ FCS})) \times 100.$$

HEPARIN BINDING ASSAY

Displacement curves of binding of [³H]-labelled heparin were performed as described in chapter 4 in order to characterise heparin binding in 28 human VSMC cultures. Cells were seeded as before at the same density as in the previous chapter and growth arrested for 72 hours as described above. The cells were then cooled to 0-4°C and washed with ice cold PBS. Pre-cooled solutions of [³H]-heparin at 10⁻⁷M with varying concentrations of unlabelled heparin (10⁻⁹M to 10⁻⁵M), were added to triplicate wells for 30 minutes and maintained on ice during the assay. (In a further 13 cell cultures specific binding was calculated using only (10⁻⁹M and 10⁻⁵M unlabelled heparin). At the end of the incubation period, cells were washed three times with ice cold PBS and solubilised in 1M NaOH overnight at room temperature. Radioactivity was

counted in a liquid scintillation counter as before. Proliferation and binding assays were performed at the same time on cells from individual cell cultures.

STATISTICAL ANALYSIS

Mann Whitney *U* test was used to compare the median heparin inhibition values for stenosed and non stenosed grafts. Spearman's rank correlation coefficient was used in the comparison between heparin inhibition and the amount of binding in individual VSMC cultures, and the point biserial correlation coefficient for analysis of dichotomous data (Dunnington et al. 1990).

MATERIALS

As used in previous chapter.

RESULTS

In order to estimate the density of [³H]-heparin binding to VSMC, at a concentration of 10⁻⁷ M, displacement curves were constructed for cell cultures from 25 patients (examples in figure 1.). Maximum binding was calculated for a further 13 cultures. In 15 of the 38 patients graft outcome data was also available (chapter 4), with binding and proliferation assays performed

concurrently. Analysis of the binding suggested a single population of binding sites (chapter 5) and showed a marked variation in [³H]-heparin binding between different VSMC cultures with the number of sites ranging from approximately from 10⁵ to 10⁶/cell. It should be noted that this does not represent maximal binding, since the concentration of labeled heparin was not saturating but rather approximated to the dissociation constant. Proliferation assays on the same cell cultures also revealed a varied response with a range of values for heparin inhibition (0- 95%, median value 42.3%). In cultures derived from the same patient a highly significant positive correlation was observed between heparin inhibition of proliferation and the density of binding of [³H]-heparin (10⁻⁷M) ($r = 0.81$; $n=38$; $p < 0.0001$,).(figure 2.). In other experiments (chapter 5) I have demonstrated that this binding site is saturable.

In the sub-group of 15 patients where binding, growth and clinical data (chapter 4) were known, 8 patients had patent grafts at the end of 1 year while stenoses had been detected in the remainder. Both heparin binding and growth inhibition by heparin were again significantly correlated with clinical outcome (point biserial correlation coefficients, $r = 0.56$, $p < 0.05$ and $r = 0.66$, $p < 0.02$). As one would anticipate from the overall patient data there was also a strong correlation between binding and growth inhibition in this sub-population ($r = 0.79$, $p = 0.001$) (figure 3.), giving a multiple correlation coefficient of 0.65 for the three parameters.

DISCUSSION

Many therapeutic agents have proved highly effective in attenuating MIH following vascular injury in animal models but this has not been predictive of success in clinical trials, which have been consistently negative (Faxon and Currie 1995). I have therefore used cultured human VSMC as a model system to investigate the control of cell proliferation. In previous studies it has been shown that cells from a given individual behave consistently over several passages, notably in terms of response to growth inhibition by heparin. Furthermore, this consistency is also seen in cells taken from different vessels in the same patient, including those from stenotic lesions themselves (Munro et al. 1994). By contrast, in confirmation of earlier observations, I have demonstrated that there is wide inter-individual diversity in responses (Chan et al. 1993). These findings support the assumption that differences in cellular response are reproducible and significant.

Heparin resistance has been shown to be a highly significant predictor for the development of subsequent stenosis (chapter 4). Obviously, the next step was to consider possible mechanisms to explain this finding and the heparin binding study was carried out for this reason. Castellot et al. first described specific binding of heparin to animal VSMC in 1985(b), followed by the observation of Resink et al (1989) that growth inhibition in rat VSMC was related to the density of heparin binding sites in the SHR model of hypertension where both parameters were reduced as compared to WKY controls. Barzu et al (1994) have shown that it is possible to isolate heparin-resistant rat VSMC by growing them

in culture in the presence of heparin for up to 15 passages. Here too growth inhibition by heparin altered in parallel with the extent of reduction in heparin binding, though this association was not found by San Antonio et al. (1993). The mechanism of heparin inhibition is itself still incompletely understood. Heparin inhibits the activation of the mitogen-activated protein kinase in intact rat VSMC but the intracellular mechanisms involved have not been defined (Ottlinger et al. 1993) and it is unclear whether internalisation of heparin is required for its antiproliferative action (see chapter 7). Since my experiments were carried out in cell monolayers the possibility also remains that it is the interaction of heparin with extracellular matrix, rather than cell-associated binding sites, that mediates its inhibitory action on these cells (Au et al. 1993). The same group has recently reported the failure of heparin to inhibit intimal hyperplasia in a primate, the baboon, while drawing attention to the existence of heparin-sensitive and insensitive pathways of mitogenesis (Geary et al. 1995). It must of course be remembered that these concentrations of heparin are far above therapeutic anticoagulant levels and these results therefore have no implications for the clinical use of heparin in stenosis or restenosis prophylaxis.

My results indicate a clear relationship between clinical outcome and in vitro heparin inhibition (see chapter 4), and between inhibition and heparin binding, while in a sub-group of patients in whom I have all three sets of data a strong correlation has been confirmed for these parameters. Furthermore, one must always be cautious about any extrapolation from in vitro cell culture to a clinical setting, bearing in mind that cell culture inevitably implies the loss of *in vivo* local and systemic regulatory mechanisms.

In conclusion, I have demonstrated for the first time a cellular marker in vascular smooth muscle which is highly predictive of subsequent stenosis in vein graft recipients and have also presented evidence on which to base the formulation of a possible mechanism. The role of extracellular matrix proteins as regulators of cell growth and differentiation is a possible mechanism which needs further investigation. These findings have several implications for future research and ultimately for clinical management. Altered responsiveness to heparin may itself be a marker for a more fundamental disturbance of growth regulation in the vascular wall, though this does not preclude the possibility that a failure to interact appropriately with glycosaminoglycans in the vessel wall has in itself an important role in the disease process. Understanding these mechanisms and how they could be modulated will contribute to better understanding of the biology of the vascular wall and may also lead to more rational and effective management of an important clinical problem, with the intriguing prospect of identifying individuals at increased risk of graft failure and ultimately reducing the likelihood of an unfavourable outcome.

Figure 1.

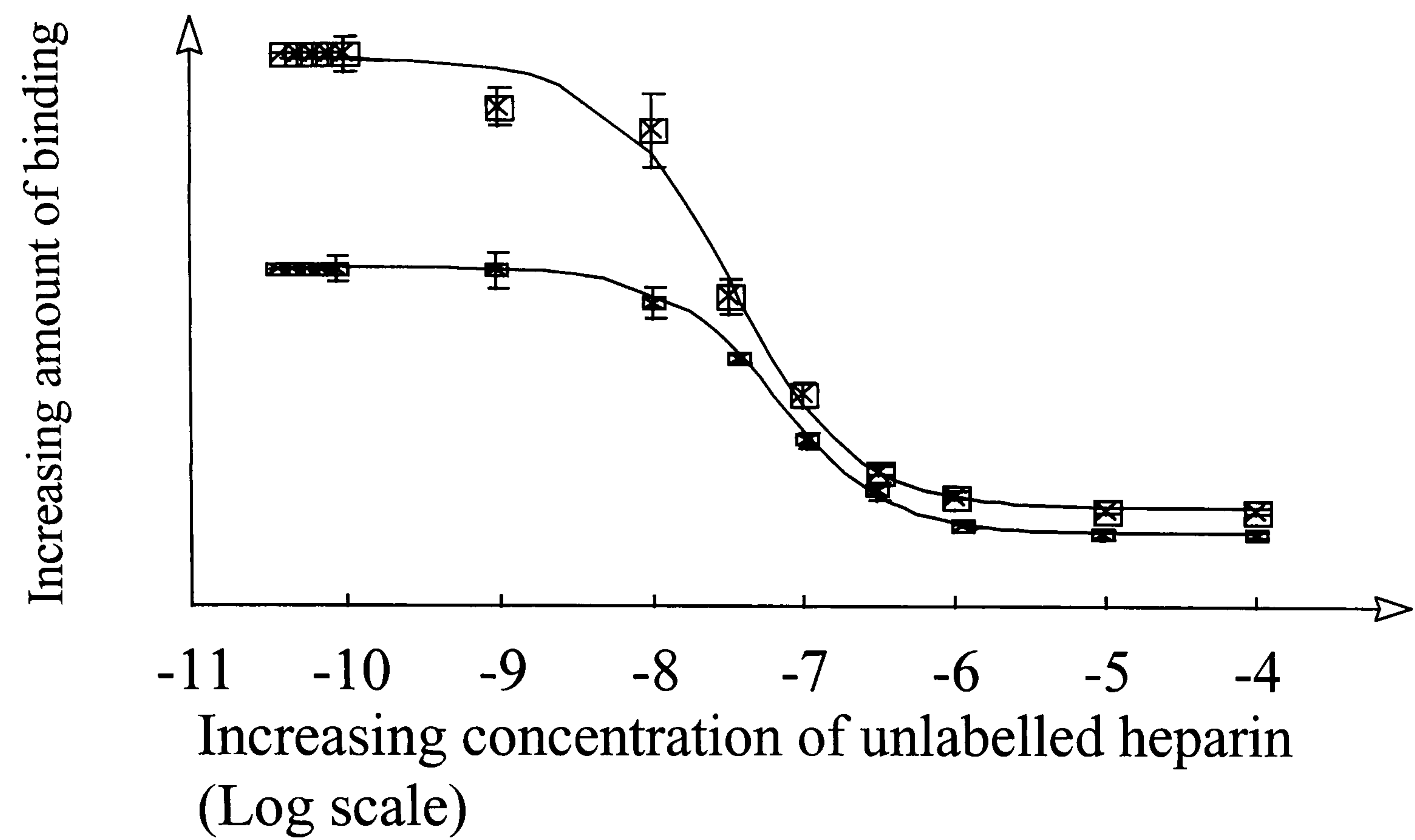


Figure 1. Representative inhibition curves of $[^3\text{H}]$ -heparin binding in a cell culture resistant to growth inhibition by heparin (<25% inhibition, circles) and a culture >75% inhibited by heparin (squares).

Figure 2.

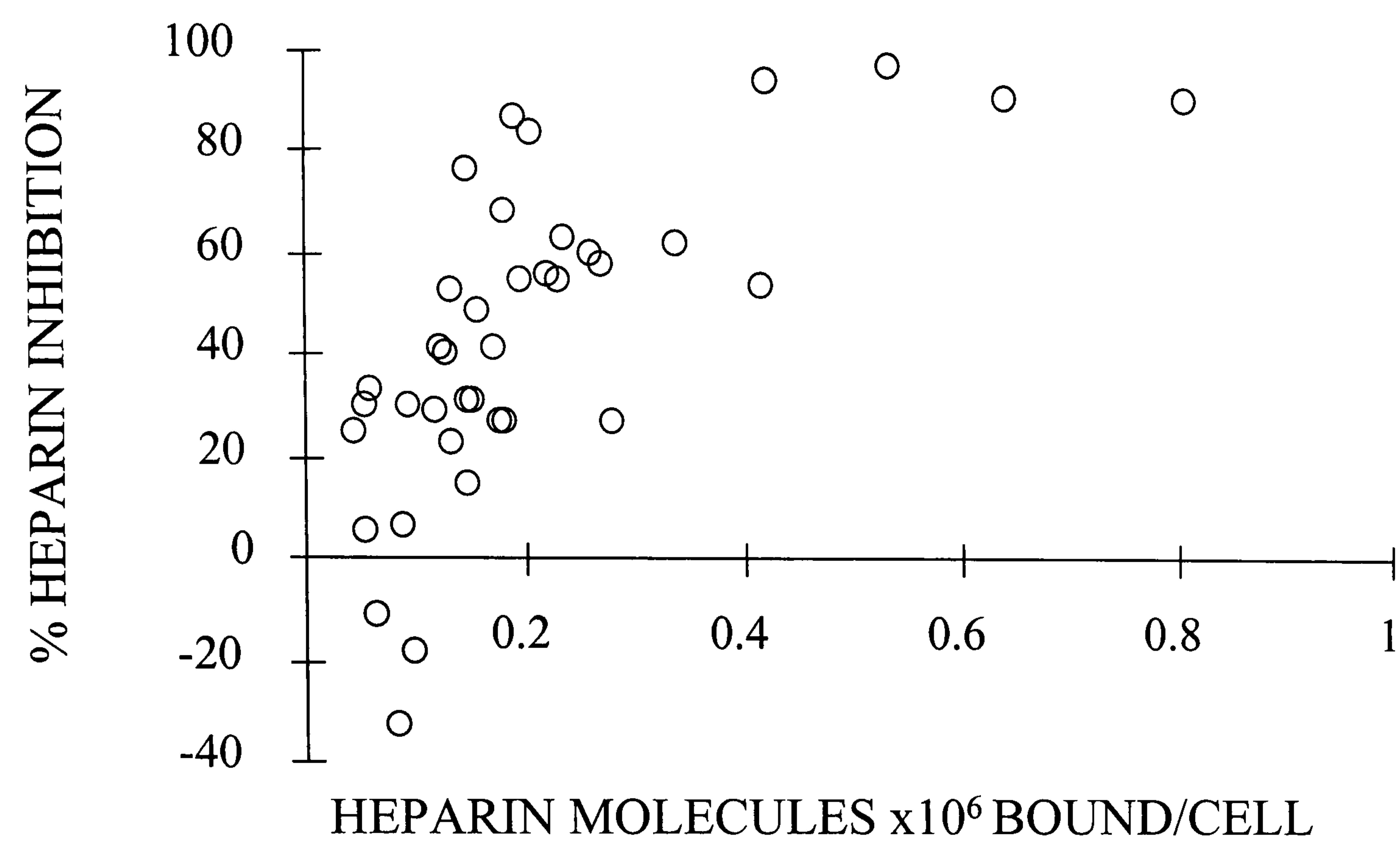


Figure 2. Relation of inhibition of vascular smooth muscle cell proliferation with [3H]-heparin binding . Each symbol represents a patient n=38.

Figure 3.

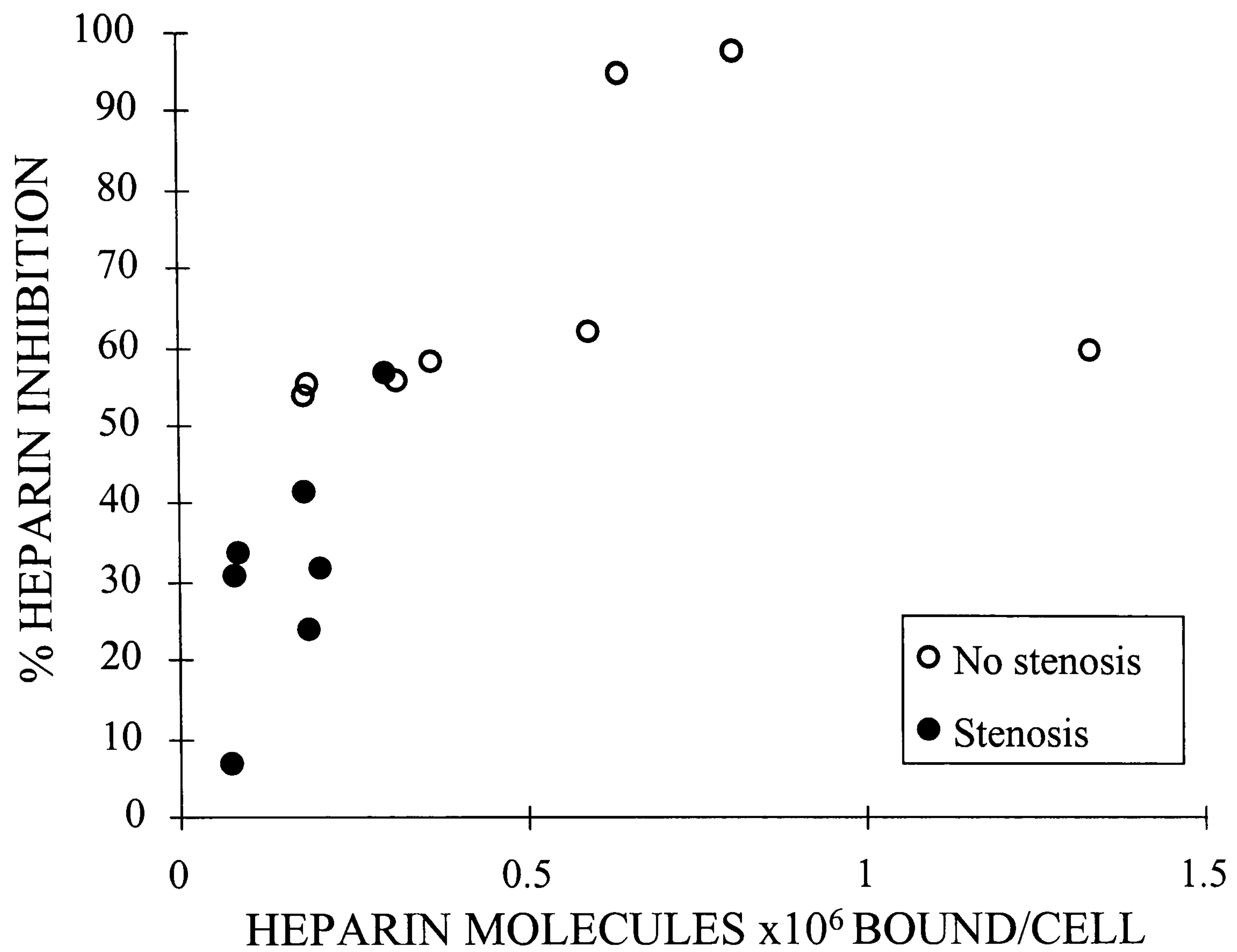


Figure 3. Relation of inhibition of smooth muscle proliferation by heparin with [³H]-heparin binding in patients with (solid symbols: n=7) and without (open symbols: n=8) infra-inguinal vein graft stenosis.

PEPTIDE GROWTH FACTORS I: THE EFFECT ON HUMAN VSMC PROLIFERATION.

INTRODUCTION

In order to further understand the effects of individual peptide growth factors on human VSMC I have examined the responsiveness of human cells stimulated with specific growth factors. This is an area that has not been extensively studied. Most work on human VSMC has been carried out using 15% foetal calf serum (FCS) and this is almost certainly the strongest growth-promoting stimulus one can use, but has some well-recognised drawbacks. There can be very large inter-batch variability in activity: more importantly, many of the growth stimulants in the serum have yet to be characterised. By contrast, most work using individual growth factors has been carried out *in vivo* using the small mammal model or *in vitro* using small mammal VSMC. I, have used a number of specific VSMC growth factors (EGF, basic FGF, IGF and PDGF AA, AB and BB) in the absence of serum and examined their growth promoting effects on human VSMC over a range of concentrations.

Saphenous vein is particularly important as a source of VSMC in studying this process as it is the most widely used conduit for coronary artery and infra-

inguinal bypass procedures and MIH as a result of VSMC proliferation and migration accounts for approximately 80% of all failures of saphenous vein grafts.

MATERIALS AND METHODS

TISSUE CULTURE

Samples of redundant long saphenous vein were obtained from twelve patients undergoing coronary artery and peripheral bypass surgery. These tissues would normally have been discarded at the time of surgery and their use conforms with the guidelines of St. Mary's Hospital Ethical Committee. All tissue was transported from theatre to laboratory as previously described.

DNA SYNTHESIS ASSAYS

Tritiated thymidine incorporation was used as a measure of DNA synthesis. Cells were seeded at a density of 9×10^4 / ml in 96 well plates (Nunc, 0.2 ml/well) and allowed to attach overnight. The 15% Foetal calf serum containing medium was removed and the cells washed twice with phosphate buffered saline (PBS without calcium or magnesium). The cells were then growth arrested for 72 hours using serum free medium. This comprised Dulbecco's

modified Eagle medium with added Insulin (10^{-6}M), transferrin ($5\mu\text{g/ml}$), Sodium selenite (10^{-7}M), Ascorbic acid ($5\times 10^{-4}\text{M}$) and 1% bovine serum albumin (Libby and O'Brien 1983). Growth stimulation was then commenced with medium containing PDGF AA, AB, BB, EGF, IGF, basic FGF and foetal calf serum as a positive control. Serum free medium with no added growth factors was used as a negative control. Sextuplicate treatments were added for each concentration of each growth factor. All treatments were carried out simultaneously. For assays performed using IGF, serum free medium with and without added insulin was used.

After 24 hours of incubation [methyl ^3H] thymidine was added ($1\mu\text{Ci/well}$, $40\mu\text{Ci/mL}$) for a further 6 hours. The experiment was terminated by washing the cells twice with trichloro acetic acid (10%, w/v) and then solubilising the cells in $200\mu\text{L}$ sodium hydroxide (1 mol/L) at 37°C for 12 hours. In order to precipitate solubilised DNA the pH was neutralised with $50\mu\text{L}$ hydrochloric acid (4 mol/L). Precipitated DNA was harvested onto glass fibre filter mats and the radioactivity counted using a Matrix 96 direct beta counter (Canberra Packard, Pangbourne England).

DRUGS

FCS was from J. Bio (Les Ulis, France). [Methyl ^3H] thymidine (925 GB/mmol) was purchased from Amersham International (Aylesbury, U.K). Recombinant PDGF isoforms (AA, AB and BB) were obtained from Boehringer Mannheim (Lewes, U.K). Insulin, sodium selenite, human transferrin, ascorbic acid and BSA purchased from Sigma (Poole, U.K). All other cell culture materials were obtained from GIBCO Life Technologies/BRL (Paisley, U.K), all other chemicals were purchased from Sigma (Poole, U.K).

STATISTICS AND DATA ANALYSIS

Data are mean $\pm$ SEM of n observations from separate cell lines. Results were compared by using analysis of variance (ANOVA) followed by least significant difference (LSD) multiple range test using commercially available shareware (Labstats 2.2, JPC Computer Services, Australia); $p < 0.05$ was considered statistically significant. Concentrations of growth factors were converted to their molar concentrations and concentration response data obtained in an individual cell line were fitted to a logistic function:

$$y = a + \frac{b-a}{\left(1 + \frac{10^c}{10^x}\right)^d}$$

where y = effect at a given concentration of growth factor, a = minimum effect, b = maximum effect, c = EC_{50} , x = $\log$ [growth factor] and d = 'pseudo Hill slope', by nonlinear regression with Excel 5.0 (Microsoft, USA) and a macro written by Alun Hughes. Because EC_{50} values are log-normally distributed, these data are $-\log EC_{50} \pm SEM$ derived from n observations.

RESULTS

All the growth factors stimulated thymidine incorporation in a dose dependent saturable fashion. The order of efficacy of the growth factors differed significantly: FCS > PDGF-AB > PDGF-BB > FGF > EGF > IGF > PDGF-AA ($p < 0.01$ by ANOVA; FCS v IGF - and + insulin $p < 0.01$ FCS v PDGF-AA) with no other significant comparisons. All EC_{50} values and percentage increases in DNA synthesis over serum are shown in table 1

The incorporation data for PDGF show that the AB and BB isoforms stimulate DNA synthesis in SMCs in a concentration dependent fashion from 1 - 100ng/mL. The concentration response curves showed saturable DNA synthesis and show that the AB and BB were approximately equally efficacious

but there was no response to the AA isoform. The EC_{50} values were 5 ng/mL and 10 ng/mL for the AB and BB isoforms respectively (figure 1).

Foetal calf serum (FCS) in a range of concentrations from 0.4-30% was as effective as PDGF AB and BB and showed concentration dependent DNA incorporation, however at the maximum concentration of 30% FCS the serum appeared to stop further incorporation. It is not clear if this was due to toxicity or an inhibitory effect of the growth factors present at high concentrations. The EC_{50} was 5% FCS which induced a 571% increase in activity compared to serum free medium (table 1).

IGF incorporation data showed concentration dependent increase in DNA synthesis and these curves were also saturable. The assay without insulin in the serum free medium resulted in more DNA synthesis than with insulin (EC_{50} $5 \pm$ ng/mL and $3 \pm$ ng/mL respectively). However, the maximum effect was only slightly greater than serum free medium and a fraction of that exerted by PDGF AB and BB and FCS (figure 2).

EGF also demonstrated concentration dependent stimulation of synthesis of DNA (range 1-100ng/mL) and was approximately 50% as efficacious as FCS. (EC_{50} 0.5 ng/mL) (figure 3).

Basic FGF was slightly more effective than EGF but less effective than PDGF AB and BB in stimulating DNA synthesis (range 1-100ng/mL). The effect was concentration dependent and reached a plateau (figure 4). (EC_{50} 0.5 ng/ml) the maximum effect was 538% greater than SFM.

DISCUSSION

Fifteen percent foetal calf serum has been shown to be the most efficacious *in vitro* stimulus to VSMC proliferation and DNA synthesis. In this study I have compared the effects of different peptide growth factors in VSMC derived from human saphenous vein and demonstrated a variety of responses. Methyl [^3H] thymidine incorporation studies showed that PDGF-AB, PDGF-BB, EGF and FGF are all less effective stimuli to DNA synthesis than FCS, however, although less effective this is not significantly different from FCS. IGF (with and without insulin in the serum free medium) and PDGF-AA on the other hand are both significantly weaker mitogens for the VSMC than FCS. These findings are in agreement with those of Grainger et al. (1994) who performed similar experiments on freshly dispersed rat aortic VSMC.

I shall discuss each peptide growth factor individually except the PDGF family which I will consider together.

Platelet derived growth factors (PDGF)

Both PDGF-AB and BB were markedly more effective than PDGF-AA in terms of DNA synthesis. This is surprising in view of the proposed affinity of receptor subtypes described (Chapter 3). In a simple model PDGF-BB might be expected to be the most effective isoform for any cellular response since it is able to bind to $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ receptor dimers whereas PDGF-AB can only bind to $\alpha\alpha$ and $\alpha\beta$ receptors. It is possible to speculate that these findings may reflect a greater efficacy of the $\alpha\beta$ compared with the $\beta\beta$ dimer as a stimulant of DNA synthesis in human saphenous vein cells, combined with a relatively greater formation of the $\alpha\beta$ dimer in the presence of PDGF-AB than PDGF-BB. In the absence of ligand PDGF, receptors exist in the membrane as monomers and because the PDGF isoform is responsible for dimerisation (Bowen-Pope et al. 1989), PDGF-AB will tend to promote association of $\alpha\beta$ as the dominant dimer form in a cell expressing both α and β receptor subtypes. By contrast, PDGF-BB may induce relatively less formation of $\alpha\beta$ dimers since many β receptors will be consumed in the formation of $\beta\beta$ dimers. Alternatively, $\beta\beta$ dimers may well just be a less effective stimulus for DNA synthesis. Further work measuring the relative concentrations of α and β receptor monomers in these cells may yield important information regarding this possibility.

PDGF isoforms have differential effects on DNA synthesis and signalling in cultured human VSMC. In view of evidence suggesting an important role for

PDGF in the pathogenesis of certain vascular diseases, including atherosclerosis, restenosis and hypertension (Bobik and Campbell 1993, Ross 1993), better understanding of the mechanism of action of the isoforms of PDGF in VSMC will have important implications for therapeutic advances in the management of such diseases (see Chapter 9).

Basic fibroblast growth factor (FGF)

Basic FGF was approximately 50% as efficacious as FCS at stimulating DNA synthesis although this difference was not statistically significant. The evidence for FGF's role in the development of MIH has been examined in Chapter 3 and from my study it can be seen that FGF does have a part to play in the stimulation of DNA synthesis and hence human VSMC proliferation. This is further corroborated by work demonstrating that human VSMCs produce a high affinity receptor for bFGF (Winkles et al. 1987) and also in two recent studies, one by Kennedy et al. (1997) who demonstrated that FGF upregulates interstitial collagenase expression in human VSMC, resulting in turnover of the extracellular matrix, an event that could facilitate VSMC migration and proliferation. In the other study by Pickering et al. (1997) the stimulation of human VSMCs with basic FGF established a phenotype in which collagen fiber production was repressed and the capacity for fiber degradation activated. This

coordinated response may be important in VSMC proliferation and migration during the early stages of atherosclerosis and restenosis.

Epidermal growth factor (EGF)

EGF was approximately 25% as effective as FCS at stimulating DNA synthesis although again this was not statistically significant. The evidence for EGF's role in the development of MIH has also been examined in Chapter 3 and found to be unclear. From my study it can be seen that EGF does have a small part to play in the stimulation of DNA synthesis and hence human VSMC proliferation. There is some confirmatory evidence that EGF may be involved in autocrine regulation of foetal VSMCs as they have been shown to both synthesize and respond to EGF (Higashiyama et al. 1994) and therefore one can speculate that EGF may be involved in autocrine regulation of adult VSMC. However, work by Mecham et al. (1991) demonstrated that there was no difference in the expression of messenger RNA for the genes for EGF receptors, between human atherosclerotic plaque derived VSMC and human saphenous vein VSMC. The role of EGF in human MIH is still unclear.

Insulin like growth factor (IGF)

It would appear that in isolation IGF has a small part to play in the stimulation of DNA synthesis, however, it is well known that in conjunction with PDGF it is a potent mitogen and chemoattractant as it has the ability to up-regulate PDGF receptor expression after injury (Pfeifle et al. 1987).

I have also demonstrated that when insulin is added to the serum free medium there is a 50 % reduction in the maximum DNA synthesis stimulated by IGF compared to when insulin is absent. Insulin and IGF are structurally related polypeptides that stimulate DNA synthesis and cellular proliferation, probably through a common pathway. Human VSMC in culture demonstrate the presence of high-affinity receptors for both these hormones. Insulin and IGF both exhibit cross-reactivity to each other's receptors but with an affinity that is 100-fold less than for the homologous receptor. However, the IGF receptor has been shown to be the common pathway for the growth effects of both insulin and IGF (Banskota et al. 1989). This may explain our findings, when comparing the effect of IGF $\pm$ insulin on DNA synthesis, as IGF and insulin may be competing with each other for the IGF receptor.

In isolation all the above growth factors have a role in stimulating DNA synthesis by VSMCs though some are more efficacious than others. However, in

the vessel wall these growth factors do not act in isolation, but will all act in concert and they will also interact with the extracellular matrix which surrounds the VSMC. The next step of this study is to examine the interaction between these growth factors and the VSMC growth inhibitor heparin which is closely structurally related to the extracellular matrix glycosaminoglycan heparan.

Table 1. Comparison of EC₅₀ values and the percentage increase in DNA synthesis over serum free medium induced by the assayed growth factors.

Mitogen	Percentage increase over SFM at EC ₅₀	EC ₅₀ in ng/ml	EC ₅₀ in pM
FCS	571%	5%	
PDGF-AB	547%	5ng/ml	185±3 pM
PDGF-BB	544%	10ng/ml	400±7 pM
FGF	538%	0.5ng/ml	28.5±2 pM
EGF	160%	0.5ng/ml	81.9±4 pM
PDGF-AA	114%	1ng/ml	34.5±2 pM
IGF-insulin	75%	5ng/ml	654±7 pM
IGF+insulin	74%	3ng/ml	393±5 pM

Figure 1

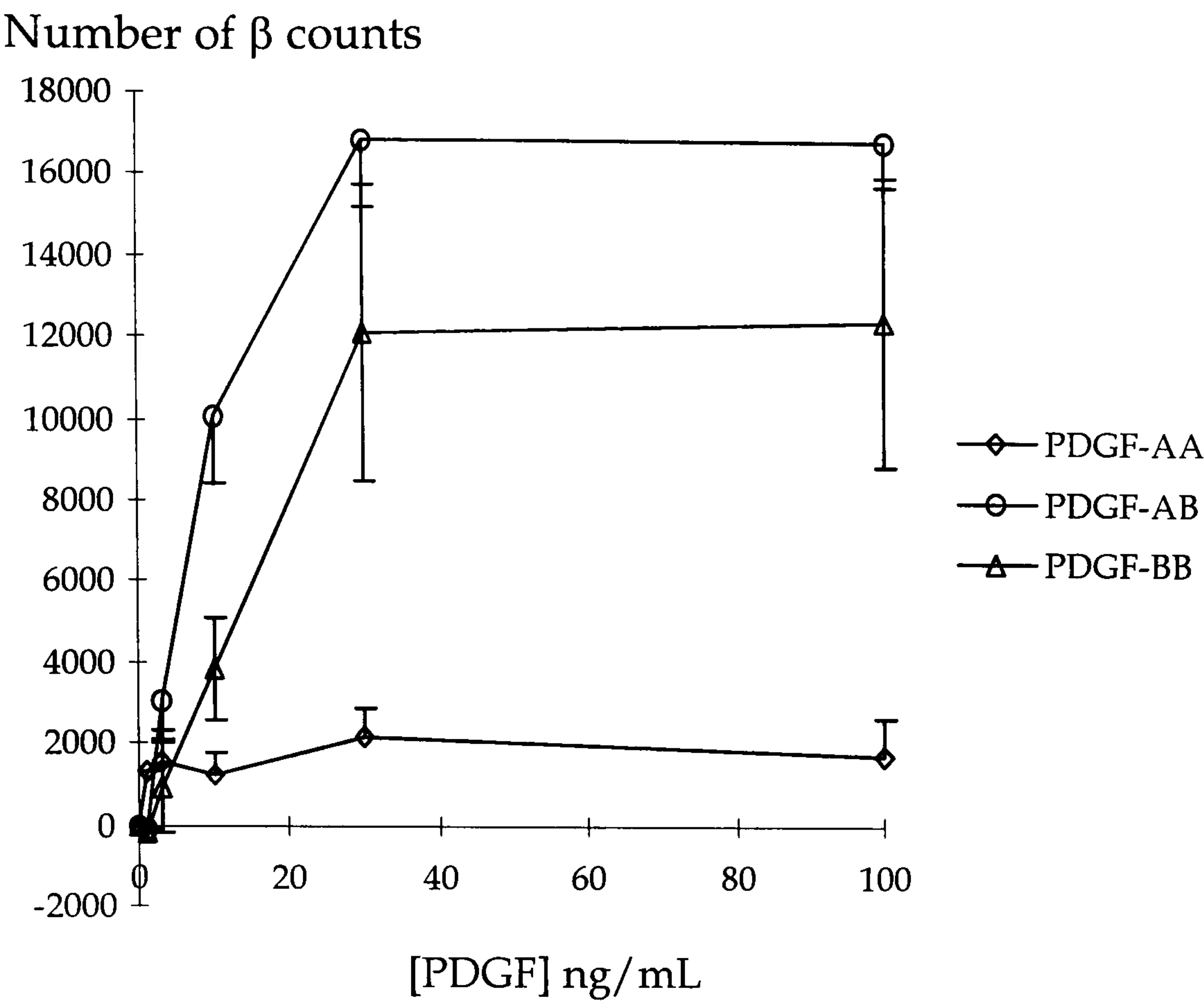


Figure 1. The effect of platelet-derived growth factor (PDGF-AA, AB and BB) on DNA synthesis in cultured human saphenous vein smooth muscle cells as determined by $[^3\text{H}]$ -thymidine incorporation. The graph shows typical concentration-response relationships for individual cell lines expressed as absolute counts of β radiation (minus the background counts for the negative control of serum free medium) and are presented as mean $\pm$ sem.

Figure 2

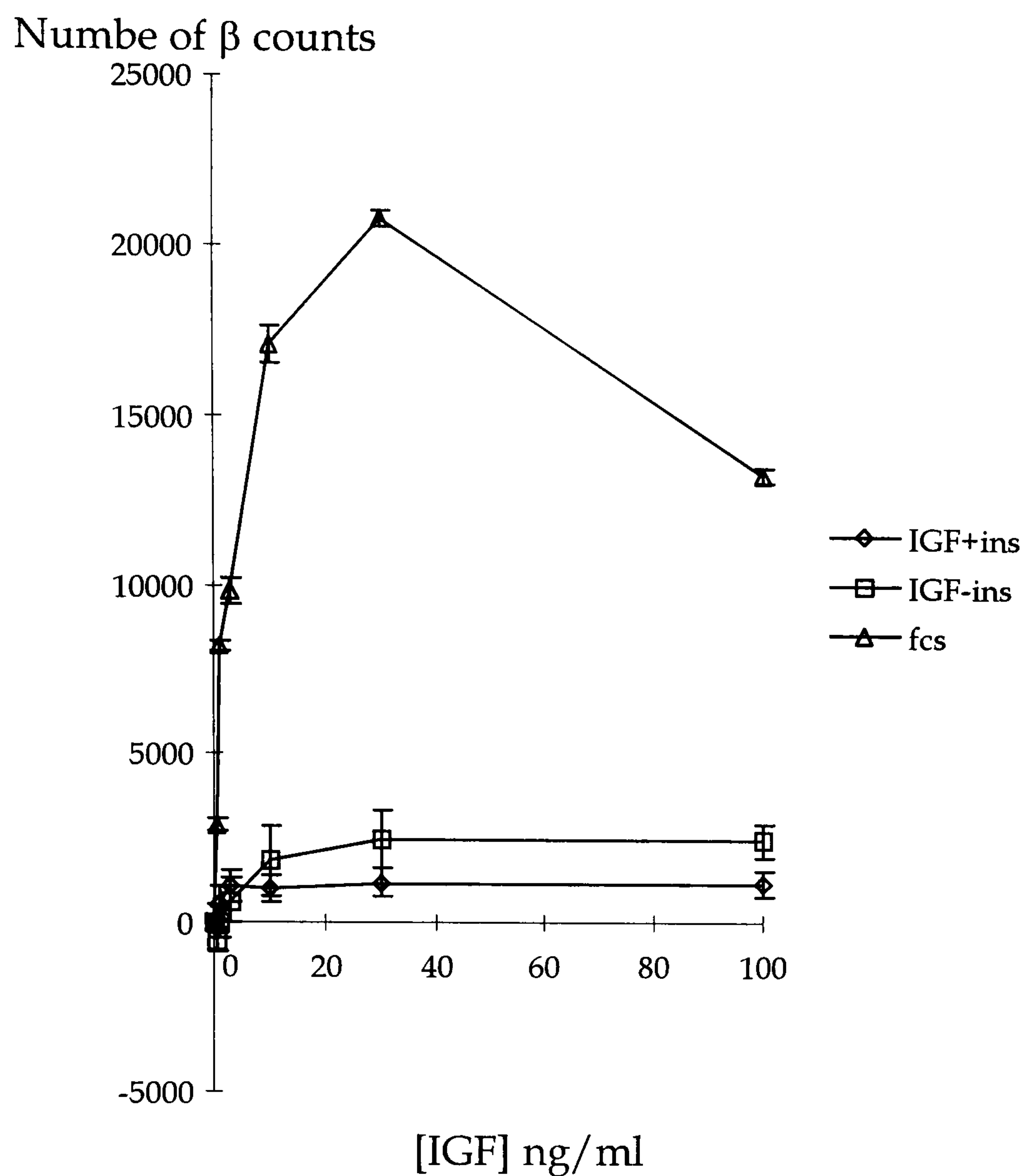


Figure 2. The effect of Insulin like growth factor (IGF), on DNA synthesis in cultured human saphenous vein smooth muscle cells determined by [^3H]-thymidine incorporation in 12 VSMC lines. The graph shows typical concentration-response relationships for individual cell lines exposed to FCS, IGF and IGF in SFM minus insulin, expressed as absolute counts of β radiation (minus the background counts for the negative control of serum free medium) and are presented as mean $\pm$ sem.

Figure 3

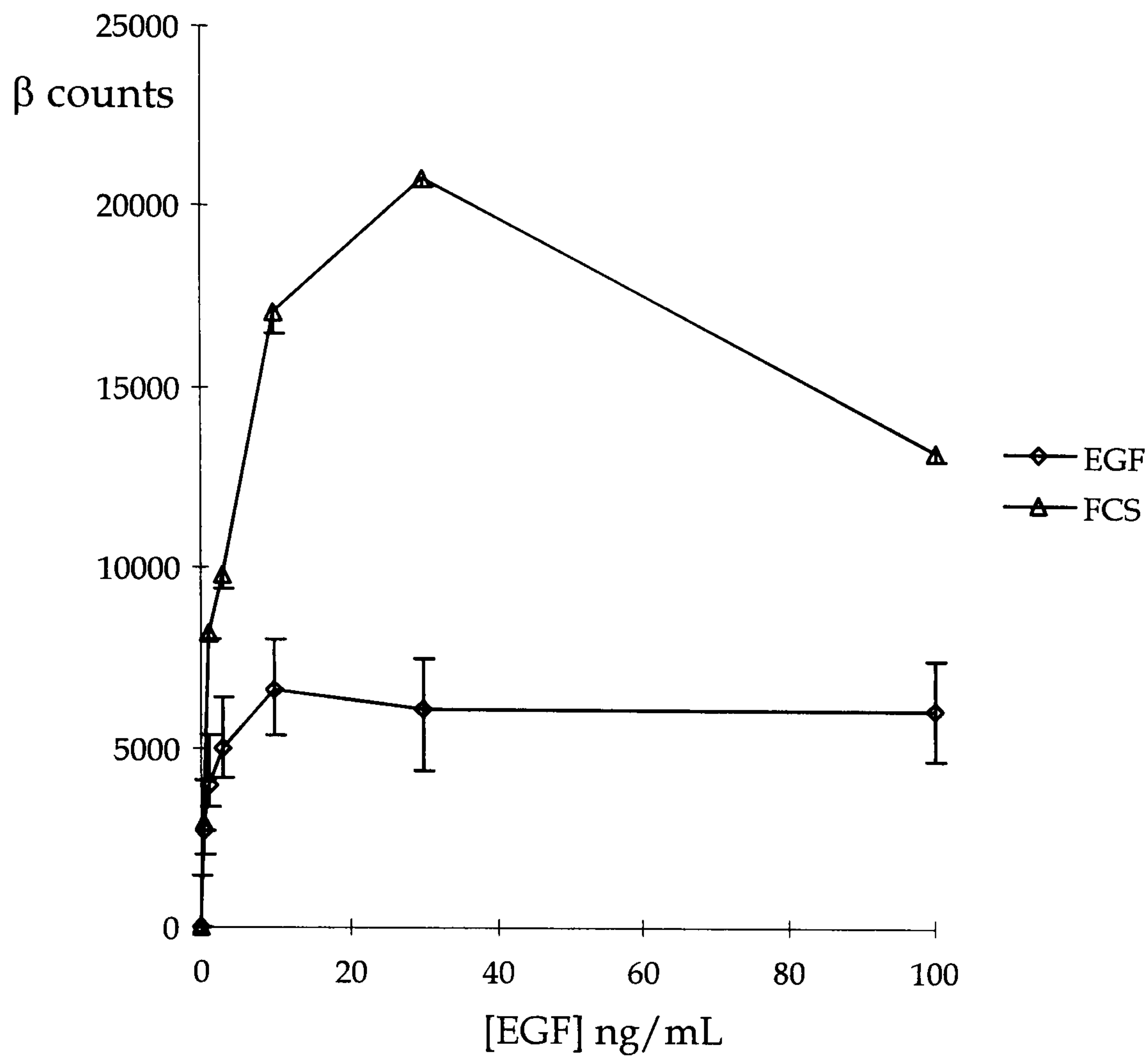


Figure 3. The effect of epidermal growth factor (EGF) on DNA synthesis in cultured human saphenous vein smooth muscle cells determined by [^3H]-thymidine incorporation in 12 VSMC lines. The graph shows typical concentration-response relationships for individual cell lines exposed to FCS, and EGF, expressed as absolute counts of β radiation (minus the background counts for the negative control of serum free medium) and are presented as mean $\pm$ sem.

Figure 4

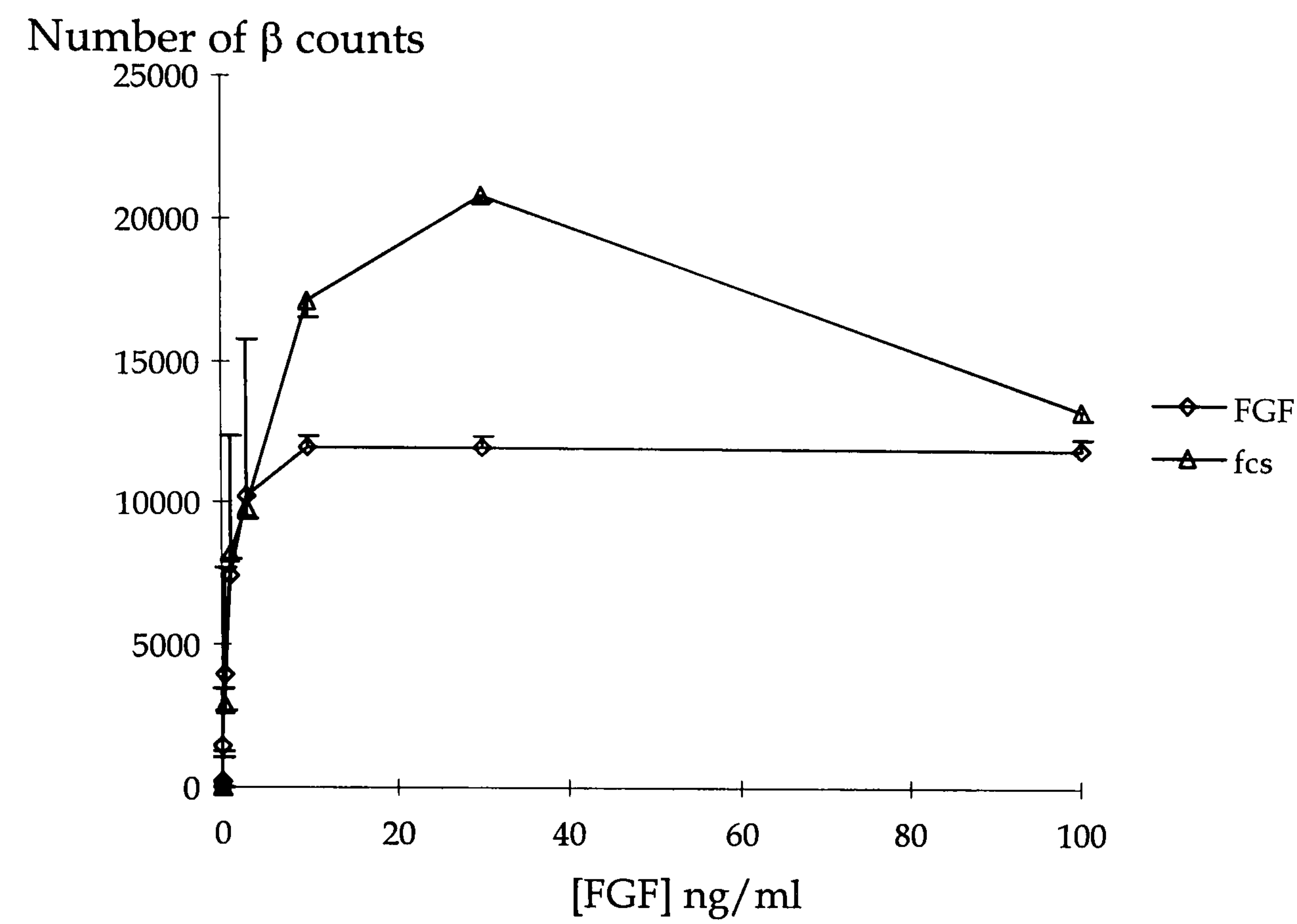


Figure 4. The effect of basic fibroblast growth factor (FGF) on DNA synthesis in cultured human saphenous vein smooth muscle cells determined by [^3H]-thymidine incorporation in 12 VSMC lines. The graph shows typical concentration-response relationships for individual cell lines exposed to FCS, and FGF, expressed as absolute counts of β radiation (minus the background counts for the negative control of serum free medium) and are presented as mean $\pm$ sem.

PEPTIDE GROWTH FACTORS II: THE EFFECT OF HEPARIN ON HUMAN VSMC PROLIFERATION.

INTRODUCTION

The growth factors discussed in the previous chapter have important modulatory roles in both VSMC and endothelial cell proliferation and migration in response to injury. Several of these growth factors are known to bind heparin (Burgess and Maciag 1989), which has been shown to prevent VSMC proliferation and migration and to limit MIH following injury (Clowes and Karnowsky 1977, Majack and Clowes 1984, Lindner et al. 1992). Specific heparan sulphate moieties have also been identified, produced by endothelial cells (Castellot et al. 1981) and by VSMC themselves, (Fritze et al. 1985) that inhibit the proliferation of VSMC. These findings suggest that cells within the vessel wall are capable of producing unique antiproliferative species of heparin like molecules that regulate the growth responses of VSMC. It has been suggested that the complexing of heparin and certain peptide growth factors is at least partly responsible for the changes in the biological effects of the growth factors (Burgess and Maciag 1989). In this study I shall examine heparin's anti-proliferative action over a range of concentrations against the EC₅₀

concentration of each growth factor as determined in the previous chapter. I will also compare the effects of the heparin binding growth factors with non binding growth factors.

MATERIALS AND METHODS

TISSUE CULTURE

Samples of redundant long saphenous vein were obtained from the same twelve patients undergoing coronary artery and peripheral bypass surgery as discussed in the previous chapter. All tissue was transported from theatre to laboratory as previously described.

DNA SYNTHESIS ASSAYS

Tritiated thymidine incorporation was used as a measure of DNA synthesis. Cells were seeded at a density of 9×10^4 / ml in 96 well plates (Nunclon, 0.2 ml/well) and allowed to attach overnight. The 15% FCS containing medium was removed and the cells washed twice with phosphate buffered saline (PBS without calcium or magnesium). The cells were then growth arrested for 72 hours using serum free medium. This comprised Dulbecco's modified Eagle

medium with added Insulin (10^{-6}M), transferrin ($5\mu\text{g/ml}$), Sodium selenite (10^{-7}M), Ascorbic acid ($5 \times 10^{-4}\text{M}$) and 1% bovine serum albumin (Libby and O'Brien 1983). Growth stimulation was then commenced with medium containing EC_{50} concentrations of the growth factors (PDGF AA (34.5 pM), AB (185 pM), BB (400 pM), EGF (82 pM), IGF (654 pM), basic FGF (29 pM) and foetal calf serum (5%) (as a positive control)) mixed with heparin in a range of concentrations from 1-1000 $\mu\text{g/mL}$. Sextuplicate treatments were added for each concentration of heparin and each growth factor. All treatments were carried out simultaneously. For assays performed using IGF, serum-free medium with and without added insulin was used.

After 24 hours of incubation [methyl ^3H] thymidine was added ($1\mu\text{Ci/well}$, $40\mu\text{Ci/mL}$) for a further 6 hours. The experiment was terminated by washing the cells twice with trichloro acetic acid (10%, w/v) and then solubilising the cells in 200 μL sodium hydroxide (1 mol/L) at 37°C for 12 hours. In order to precipitate solubilised DNA the pH was neutralised with 50 μL hydrochloric acid (4 mol/L). Precipitated DNA was harvested onto glass fibre filter mats and the radioactivity counted using a Matrix 96 direct beta counter (Canberra Packard, Pangbourne England).

DRUGS

As in previous chapter, Heparin (Paynes & Byrne, U.K.).

STATISTICS AND DATA ANALYSIS

As in previous chapter.

RESULTS

Heparin was able to inhibit the mitogenic effects of the growth factors in the following order IGF> FGF> FCS> PDGF-AA> PDGF-AB> EGF> PDGF-BB (Figure 1), when used at their EC₅₀ concentration. This was achieved in a dose dependent fashion with increasing effect from 1-1000 µg/mL. Using FCS as a positive control there was no significant difference in heparin's anti-proliferative action for IGF, FGF and PDGF-AA stimulated DNA synthesis ($p > 0.1$: ANOVA). However, heparin was unable to prevent DNA synthesis when VSMC were stimulated by PDGF-AB, PDGF-BB and EGF when compared to FCS ($p < 0.001$ by ANOVA; FCS v PDGF-AB, $p < 0.005$; FCS v EGF and $p < 0.01$; FCS v PDGF-BB).

Heparin inhibited 5% FCS in a dose dependent fashion with maximum inhibition of 57% compared to the control value for DNA synthesis (that of serum free medium). IGF (5ng/mL) induced DNA synthesis was also inhibited

by heparin with a maximum inhibition of 75%. PDGF-AA's ability to stimulate DNA synthesis was inhibited by heparin with a maximum inhibition of 68%. All the above mitogens were weak stimuli for DNA synthesis (except FCS) (Chapter 7), however FGF (0.5ng/mL), relatively a more potent stimulus for VSMC DNA synthesis was also substantially inhibited (56%) (Table 1).

By contrast, DNA synthesis induced by EGF, and both PDGF-AB and BB was not significantly inhibited by heparin. Of interest at low concentrations of heparin and EGF, PDGF-AB and BB there paradoxically appeared to be more DNA synthesis than in the absence of heparin. The maximum inhibition for the growth factors EGF, PDGF-AB and BB was 4%, 12% and -8% respectively (Table 1).

DISCUSSION

I have demonstrated that human VSMC proliferation *in vitro* is strongly influenced by PDGF-AB and BB, and to a significant but lesser extent by EGF, FGF and IGF (Chapter 8). PDGF, FGF and EGF bind to specific receptors on the surface of the VSMC (Williams et al. 1982, Bowen-Pope and Ross 1982, Bhargava et al. 1979). After receptor occupancy, a variety of intracellular changes occur including increased tyrosine kinase activity, phospholipid

degradation, specific m-RNA synthesis and specific protein synthesis (Stiles 1983). Some or all of these events may be crucial in DNA synthesis.

Certain of the growth factors assayed are known to bind heparin in the extracellularly (e.g. EGF and FGF) and therefore, a possible method of action of heparin is that by binding the growth factor it prevents it from interacting with its receptor. If this were the case one would expect EGF and FGF to be substantially inhibited by the increasing concentrations of heparin in the assay as the heparin bound more and more growth factor. However, this is not the case: FGF's ability to stimulate DNA synthesis was easily overcome by the addition of heparin to the assay, yet EGF stimulated DNA synthesis was significantly resistant to the antiproliferative action of heparin. This would suggest that heparin is acting via an alternative route to prevent DNA synthesis in the VSMC.

It is known that heparin like molecules can exert their inhibitory potential by stopping VSMC at the G_0/G_1 part of the growth cycle. Castellot (1985a) found that heparin treated VSMC were stalled in G_0/G_1 as early as 28 hours after heparin addition (within the time course of this experiment). Heparin also blocks thymidine and uridine uptake but not amino acid uptake into VSMC within 2 hours of exposure (Castellot et al. 1985a). These results suggest that heparin-like molecules inhibit proliferation by preventing serum mitogens from promoting the G_0/G_1 to S phase transition of VSMC.

Castellot et al. (1985b) has also demonstrated that VSMC possess a specific high affinity receptor for heparin on their cell surface and are capable of internalising this complex carbohydrate. The rate of uptake was biphasic, featuring an initial rapid entry of heparin into the cell followed by a slow, steady internalisation of heparin for several days. Experiments using fluorescent labeled heparin demonstrated that this initial internalisation was by receptor mediated endocytosis.

It can be seen from chapter 5 that there is indeed specific binding of heparin to the human VSMC and as we have also demonstrated a direct correlation between binding and anti-proliferative action it seem likely that heparin may be inhibiting VSMC growth at an intracellular location.

Given these findings I think it is possible that heparin blocks the mitogenic activity of specific growth factors involved in the G_0/G_1 to S phase transition of VSMC and not just by an extracellular interaction. Further work to examine this is currently ongoing (personal communication A Hughes).

Figure 1

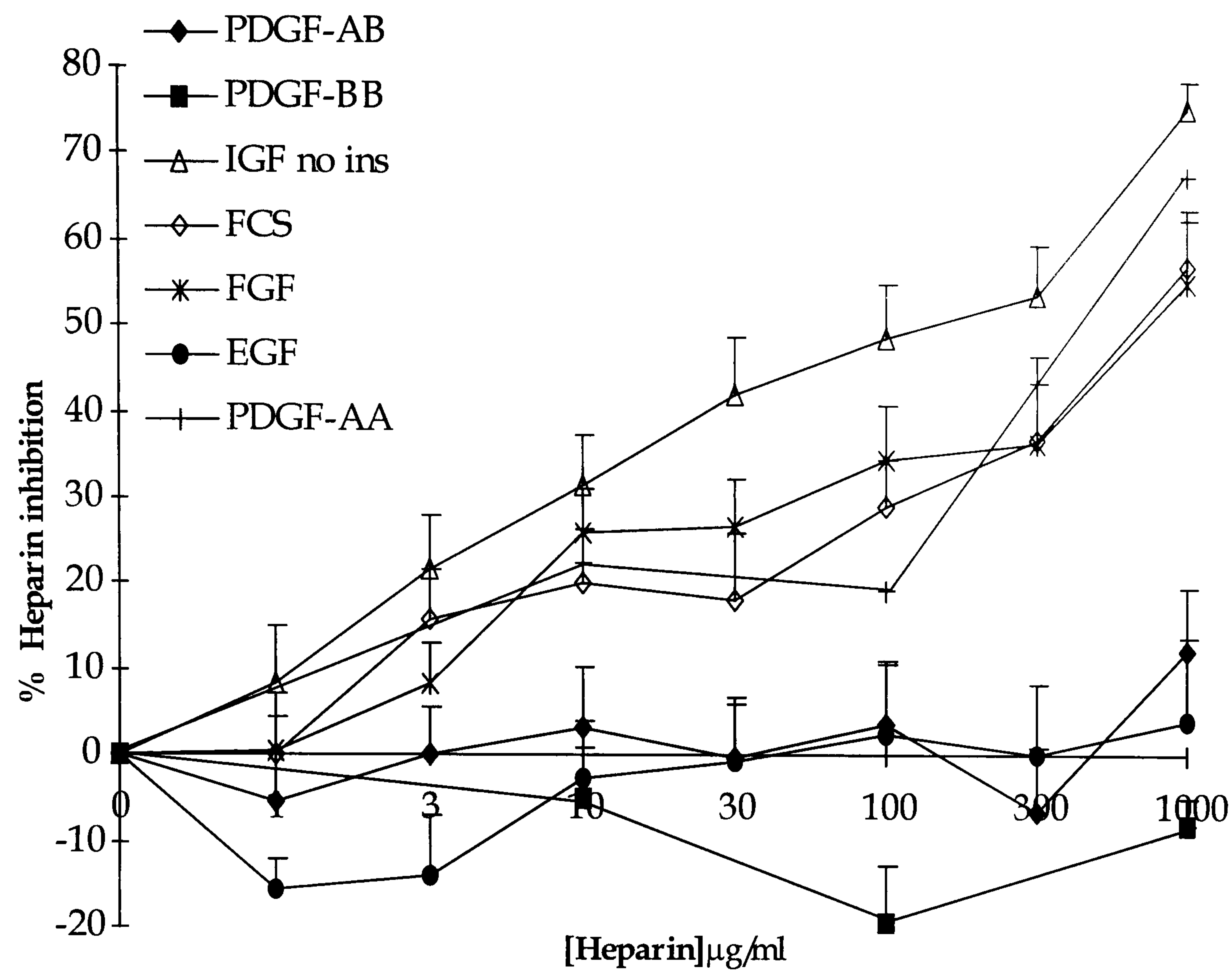


Figure 1. The effect of heparin (1- 1000 $\mu\text{g}/\text{mL}$) on DNA synthesis in cultured human saphenous vein smooth muscle cells determined by $[^3\text{H}]$ -thymidine incorporation in 12 VSMC lines (for each growth factor). The graph shows the concentration-response relationships for individual growth factors exposed to heparin as a percentage of the maximum stimulation of DNA synthesis (by each growth factor) achieved in the absence of heparin, presented as mean $\pm$ sem.

Table 1. Percentage inhibition by heparin (1000 µg/mL). Figures marked* indicate growth factors that showed a significant resistance to heparin.

Mitogen	Maximum percentage heparin inhibition	EC ₅₀ in ng/ml
FCS	57%	5%
PDGF-AB	12%*	5ng/ml
PDGF-BB	-8%*	10ng/ml
FGF	56%	0.5ng/ml
EGF	4%*	0.5ng/ml
PDGF-AA	68%	1ng/ml
IGF-insulin	75%	3ng/ml

PEPTIDE GROWTH FACTORS III: THE CO-ORDINATION OF MIGRATION AND PROLIFERATION BY PDGF ISOFORMS IN HUMAN VSMC.

INTRODUCTION

It can be seen from the preceding two chapters that although PDGF is one of many growth stimulants it is the dominant mitogen for VSMC proliferation. There is also strong evidence that it has an important role to play in VSMC migration. Studies using animal injury models have shown that infusion of PDGF-BB promoted intimal thickening and migration of VSMC from the media to the intima (Jawien et al. 1992). Furthermore, an antibody against PDGF reduced MIH (Ferns et al. 1991). Similarly in vascular organ culture models, including human saphenous vein, PDGF has been associated with intinally directed VSMC migration and proliferation (Soyombo et al. 1993).

Knowledge of the actions of PDGF on VSMC proliferation and migration in a model system would be useful in interpreting the events observed in these more complex in vivo situations. In this study I have therefore, investigated the effects of PDGF-AA, AB and BB on human saphenous vein VSMC chemotaxis and proliferation.

MATERIALS AND METHODS

TISSUE CULTURE

Samples of redundant long saphenous vein were obtained from the same twelve patients undergoing coronary artery and peripheral bypass surgery as in the previous chapter. All tissue was transported from theater to laboratory as previously described.

DNA SYNTHESIS ASSAYS

Tritiated thymidine incorporation was used as a measure of DNA synthesis as described in chapter 7.

MIGRATION ASSAYS

Confluent monolayers of SMCs were washed twice in PBS and briefly exposed to a trypsin EDTA solution (0.25% (w/v) in 1mM EDTA in PBS). Neutralisation of the trypsin was achieved by addition of 8 mL of culture medium. The resultant cell suspension was then centrifuged (1000 r.p.m., 10 minutes) and resuspended in serum free medium (SFM) supplemented with bovine serum albumin (1 mg/mL) at a density of 2.25×10^5 cells/mL.

Migration assays were carried out in blind well chemotaxis chambers (modified Boyden chambers). The upper and lower compartments were separated by 13 mm diameter gelatin coated polycarbonate filters with 8 μ m pores. 0.4 ml of cell suspension was added to the upper compartment and 0.3 mL of the relevant control or chemoattractant was added to the lower compartment unless otherwise stated. Migration was allowed to proceed for 5 hours in a humidified atmosphere of 5% CO₂ at 37°C. Cells were then fixed in absolute alcohol and stained for 15 minutes with toluidine blue (65 mMol/L). Cells on the upper side of the filters were removed, leaving the cells on the underside of the filters for counting. Cells in two fields of view (x20 magnification) on duplicate filters were counted.

MATERIALS

Recombinant PDGF isoforms (AA, AB and BB) were obtained from Boehringer Mannheim (Lewes, U.K). All cell culture materials were obtained from Gibco Life Technologies (Paisley, UK). Polycarbonate filters were from Poretics (Livermore, USA). All other chemicals were purchased from Sigma (Poole, UK).

STATISTICS AND DATA ANALYSIS

Data are presented as mean $\pm$ standard error of the mean (SEM) and results were analysed using Friedman's non-parametric test for repeated measures analysis of variance (ANOVA) followed by Conover's multiple range test for individual differences if the results of ANOVA were significant. A value of $p < 0.05$ was considered significant. EC_{50} values for DNA synthesis were those calculated from chapter 8.

RESULTS

DNA SYNTHESIS

The results of the thymidine DNA incorporation experiments are those reported in chapter 8, however because of their independent relevance to this chapter I shall repeat them. The incorporation data (figure 1) show that the AB and BB isoforms of PDGF were able to concentration-dependently stimulate DNA synthesis in VSMCs derived from saphenous vein. The concentration-response curves were saturable and show that the AB and BB isoforms were approximately equally efficacious but there was no response to the AA isoform, which was not significantly different from SFM. The EC_{50} values were 5 ng/mL and 10 ng/mL for the AB and BB isoforms respectively.

CHEMOTAXIS

The effect of the AB and BB isoforms on migration were completely different from their effects on DNA synthesis. The concentration-response relationships to each isoform in a typical cell line are shown in figure 2. PDGF-AA failed to elicit any significant migration over the range 0.1-100 ng/mL and surprisingly the AB isoform produced only a modest response. In all these experiments the responses to 15% FCS and SFM were used as positive and negative controls respectively (figure 3). All the cell lines used in this study responded strongly to FCS indicating that the lack of chemotaxis in response to PDGF-AA and PDGF-AB was not due to a lack of viability of the cells.

By contrast, PDGF-BB was able to potently stimulate chemotaxis to a level comparable to FCS (figure 3). However, the response to this isoform consisted of a biphasic curve with an extremely sharp peak (figure 2) which reached a maximum value at 1 or 2 ng/mL in three of four cell lines examined and 5-10 ng/mL in one cell line. The responses at 0.1 and 1 ng/ml were $29 \pm 11\%$ and $76 \pm 17\%$ (mean $\pm$ SEM) of the peak response for a given cell line which means that the concentration of PDGF-BB required to stimulate half maximal chemotaxis was in the range 0.1-1 ng/ml. At concentrations of 10-100 ng/mL PDGF-BB the mean response was not significantly different from SFM, indicating that there was little or no directed migration occurring across 90 % of the experimentally tested range. For cells incubated in 100 ng/mL PDGF-BB, trypan blue exclusion levels at $t=0$ and 5 hours were not significantly different (data not shown), which was used as a further verification of cell viability. In

addition, thymidine incorporation at this concentration was not impaired (figure 1).

To highlight the different concentration ranges over which the proliferative and chemotactic effects of PDGF-BB operated, typical DNA synthesis and chemotaxis responses were superimposed on the same plot (figure 4). This overall profile, together with the accompanying schematic diagram, are referred to in the discussion section.

The BB isoform binds to both PDGF- α and PDGF- β receptors. There is evidence that α receptors negatively regulate migration in some VSMC types (Koyama et al. 1992 and 1994). It was therefore appropriate to investigate whether activation of PDGF- α receptors could account for the downturn in the PDGF-BB concentration-response curve. In these experiments, cells stimulated by PDGF-BB (2 ng/mL) were also exposed to PDGF-AA (1 and 100 ng/mL) in both the upper and lower compartments, following the protocol of Koyama et al. (1994) to separate chemotactic from chemokinetic effects. The results are summarised in figure 5. PDGF-AA at a concentration of 1 ng/mL had no effect on PDGF-BB induced chemotaxis. At a concentration of 100 ng/mL PDGF-AA the mean response to PDGF-BB was reduced by approximately 30 % regardless of whether the AA isoform was in the upper or lower compartment but this reduction did not reach statistical significance. PDGF-BB alone however, at a concentration of 100 ng/mL produced a response comparable to SFM. These results suggest that PDGF- α receptors may display some inhibitory properties

in these cells but they do not account for the abolition of the chemotactic response at higher concentrations of PDGF-BB.

In fact, the results above suggest that the majority of the downturn of chemotaxis at high PDGF-BB concentrations could originate from PDGF- β receptors themselves. It was therefore relevant to test the effect of the AB isoform on the ability of the BB isoform to induce chemotaxis. Cells stimulated by PDGF-BB (2 ng/mL) were incubated with or without PDGF-AB (100 ng/mL). As can be seen from figure 6, PDGF-AB inhibited PDGF-BB induced chemotaxis to such an extent that the response was no longer significantly different from SFM. Since PDGF-AB is thought to cause activation of $\alpha\alpha$ and $\alpha\beta$ dimers, the larger inhibitory effect of PDGF-AB than PDGF-AA on PDGF-BB induced chemotaxis was probably due to the $\alpha\beta$ dimers, supporting a role for PDGF- β receptors.

A further remaining question concerned whether the BB isoform at high concentrations was simply non-stimulatory or actually inhibitory for chemotaxis. To test this, cells were exposed to FCS (15%) with or without added PDGF-BB (100 ng/mL). Figure 7 shows that this concentration of PDGF-BB was able to abolish FCS induced chemotaxis. This indicated that there was a powerful net inhibitory signal activated by PDGF-BB which predominated over the positive signals induced by the many components of FCS.

DISCUSSION

This study reports the heterogeneity and complexity of responses to the three isoforms of PDGF in terms of their ability to stimulate DNA synthesis and chemotaxis. The AB and BB isoforms were very effective in terms of eliciting DNA synthesis but the AA isoform was non-stimulatory. In terms of chemotaxis PDGF-BB induced a large response, PDGF-AB a weak response and PDGF-AA no response. The peak response to PDGF-BB was not significantly different from the response to 15% FCS.

The form of the PDGF-BB chemotactic concentration-response relationship, though, was markedly different from that of the proliferative response and the concentrations required to stimulate the two effects differed by between one and two orders of magnitude (half peak chemotactic response was at <1 ng/mL whereas EC_{50} for DNA synthesis was 10 ng/mL). This observation supplies evidence that the signalling pathways involved in PDGF induced growth and migration are divergent. This is illustrated in figure 4a in which DNA synthesis and migration concentration-responses to PDGF-BB are superimposed. The plot highlights a region in which migration but not proliferation occurs and another region in which the opposite conditions prevail. A recent report (Abedi et al. 1995) using rabbit aortic SMCs has shown that PDGF-BB induced maximal tyrosine phosphorylation of p125 focal adhesion kinase at a concentration of 2.5 ng/mL which correlated with the concentrations required to induce migration. However, stimulation of the growth-associated molecules phosphatidylinositol-3-kinase, phospholipase C γ and p120 GTPase activating protein (GAP) was

achieved only at concentrations of 5-25 ng/mL. These results fit very well with the human saphenous vein VSMC proliferation and migration responses reported here. Interestingly an earlier report using CHO-R18 cells demonstrated that GAP suppressed PDGF induced chemotaxis (Kundra et al. 1994), which suggests one potential mechanism to account for the abolition of migration in the concentration range which induces growth.

Figure 4b also suggests a means by which PDGF could participate in the coordination of cell movement and proliferation. Consider a simple system consisting of a source of PDGF-BB (figure 4b). This would produce a concentration gradient of the growth factor decreasing away from the source. Cells at some distance from the source would be exposed to a small concentration of PDGF-BB sufficient to induce chemotaxis but not proliferation. The cells would migrate towards the source of PDGF-BB and in doing so ascend the concentration gradient. At higher concentrations they would receive a signal to stop migrating and receive a signal to start proliferating. Thus when the proliferation and migration data are combined to produce an overall response profile this simple model reveals that the PDGF receptors effectively encode a local positional sense in migrating cells. Such a property would be desirable in situations such as wound healing, where the wound area would be irregular and highly variable from one occurrence to another and so could not be coded for at the level of the gene. The other PDGF isoforms could play roles in this system via their direct effects on PDGF-BB induced chemotaxis and by altering the pattern of activated receptor dimer combinations.

This model could be extended to include other molecules such as transforming growth factor- β (Janat and Liao 1992, Stouffer and Owens 1994) and basic FGF (Schöllman et al. 1992) which (in addition to PDGF itself) have been shown to alter the expression of the PDGF peptide chains and receptor subtypes (Sjolund et al. 1990). Alterations in PDGF isoform and receptor ratios could encode for a wide range of positional instructions according to the situation. Furthermore, it can be envisaged that imbalances in receptor levels could lead to misplacement of proliferating cells. It is also worth emphasizing that VSMC from different human vessels may have different response profiles. This could have implications in situations such as vein grafts where two populations of smooth muscle and endothelial cells try to co-ordinate in healing a wound.

Figures 3 and 7 taken together are striking because they demonstrate that peak PDGF-BB induced chemotaxis was comparable with that of 15 % FCS but at high concentrations it was able to completely negate FCS induced chemotaxis. These findings implicate a dominant role for PDGF in VSMC migration and imply that the simple model described above may have relevance in more complex situations. The AB isoform was also able to produce an inhibitory chemotactic signal at high concentrations and, weakly, the AA isoform as well. This suggests that the PDGF- β receptors themselves activated the negative signal at higher concentrations. An additional possibility is that $\alpha\beta$ dimers (which can be formed by PDGF-AB and PDGF-BB) were responsible. To resolve this issue antibodies directed against PDGF- α receptors will need to be used.

The degree of tyrosine phosphorylation is often used as a marker for the level of activity of many signaling molecules, including the PDGF receptors. It has recently been observed in Swiss 3T3 cells that stimulation with high levels of PDGF results in a reduction in the level of tyrosine phosphorylation of certain molecules, including focal adhesion kinase (FAK), (Rankin et al. 1994) a protein thought to be intimately involved in the promotion of cell migration. High concentrations of PDGF also produced a downturn in the migratory response. However in rabbit aortic VSMC which did not exhibit a downturn, the level of FAK tyrosine phosphorylation was sustained at high PDGF concentrations (Abedi et al. 1995). A study by Clunn et al. (1997), using human VSMC, displayed peak PDGF-BB induced tyrosine phosphorylation of several bands at concentrations of growth factor which parallel those which produce peak chemotactic responses, and reduced tyrosine phosphorylation at high concentrations of PDGF-BB. Most prominent amongst these were bands at approximately 80-90 kDa and 100-120 kDa. Other bands (including a band at approximately 180 kDa, presumably the PDGF- β receptor) displayed consistently elevated levels of tyrosine phosphorylation at high concentrations of PDGF-BB, similar to the sustained DNA synthesis response. These patterns of phosphorylation could provide clues as to which tyrosine phosphorylated proteins are crucial in the control of VSMC migration. Work is currently underway aimed at identifying these molecules and the mechanisms underlying the reduction in tyrosine phosphorylation (personal communication from G Clunn 1998).

Figure 1

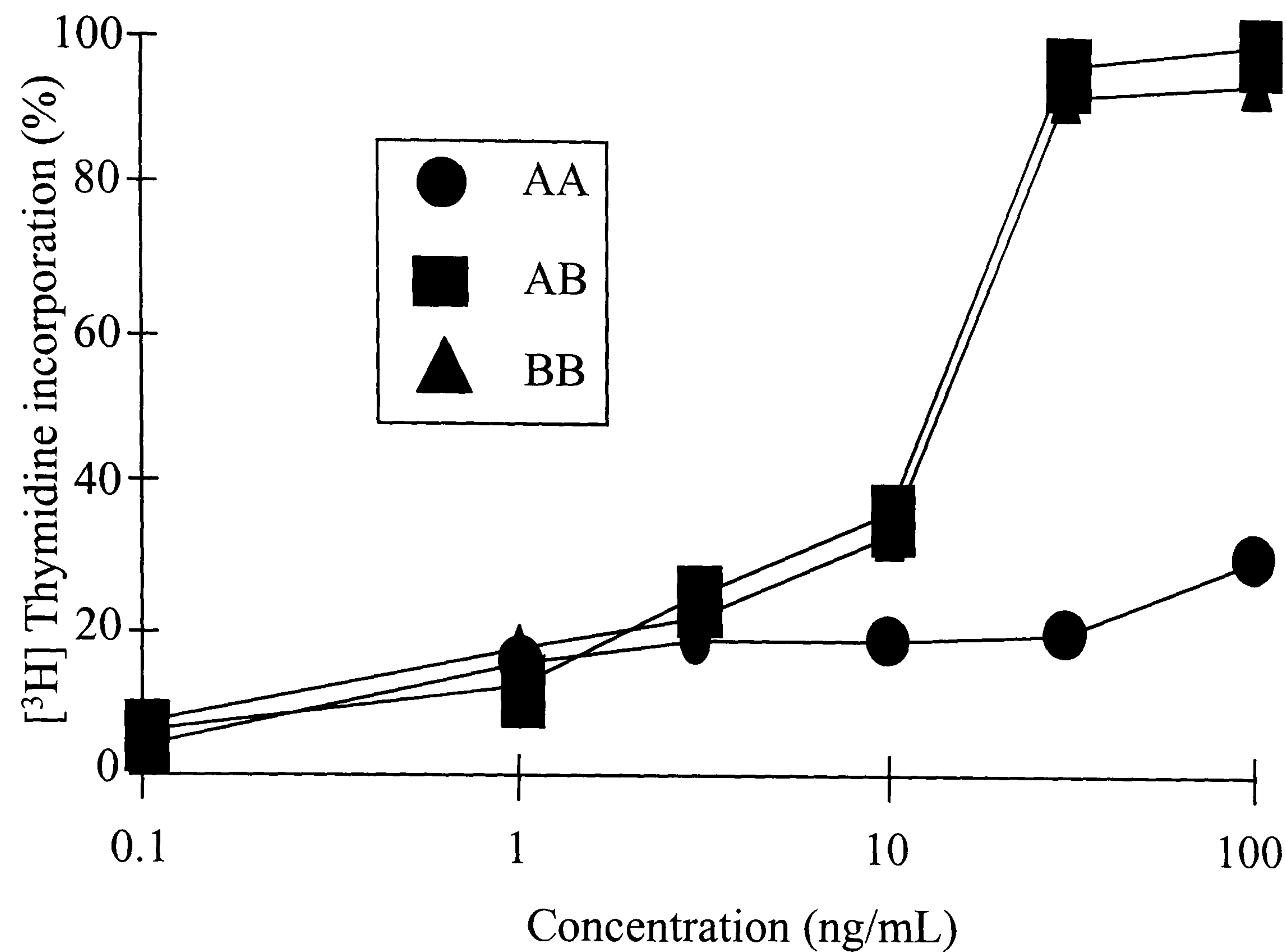


Figure 1. The effect of platelet-derived growth factor (PDGF)-AA, AB and BB on DNA synthesis in cultured human saphenous vein smooth muscle cells as determined by [3H]-thymidine incorporation. The graph shows typical concentration-response relationships for individual cell lines expressed as a percentage of maximal [3H]-thymidine incorporation in response to PDGF-BB and are representative of experiments carried out on 6-8 separate cell lines.

Figure 2.

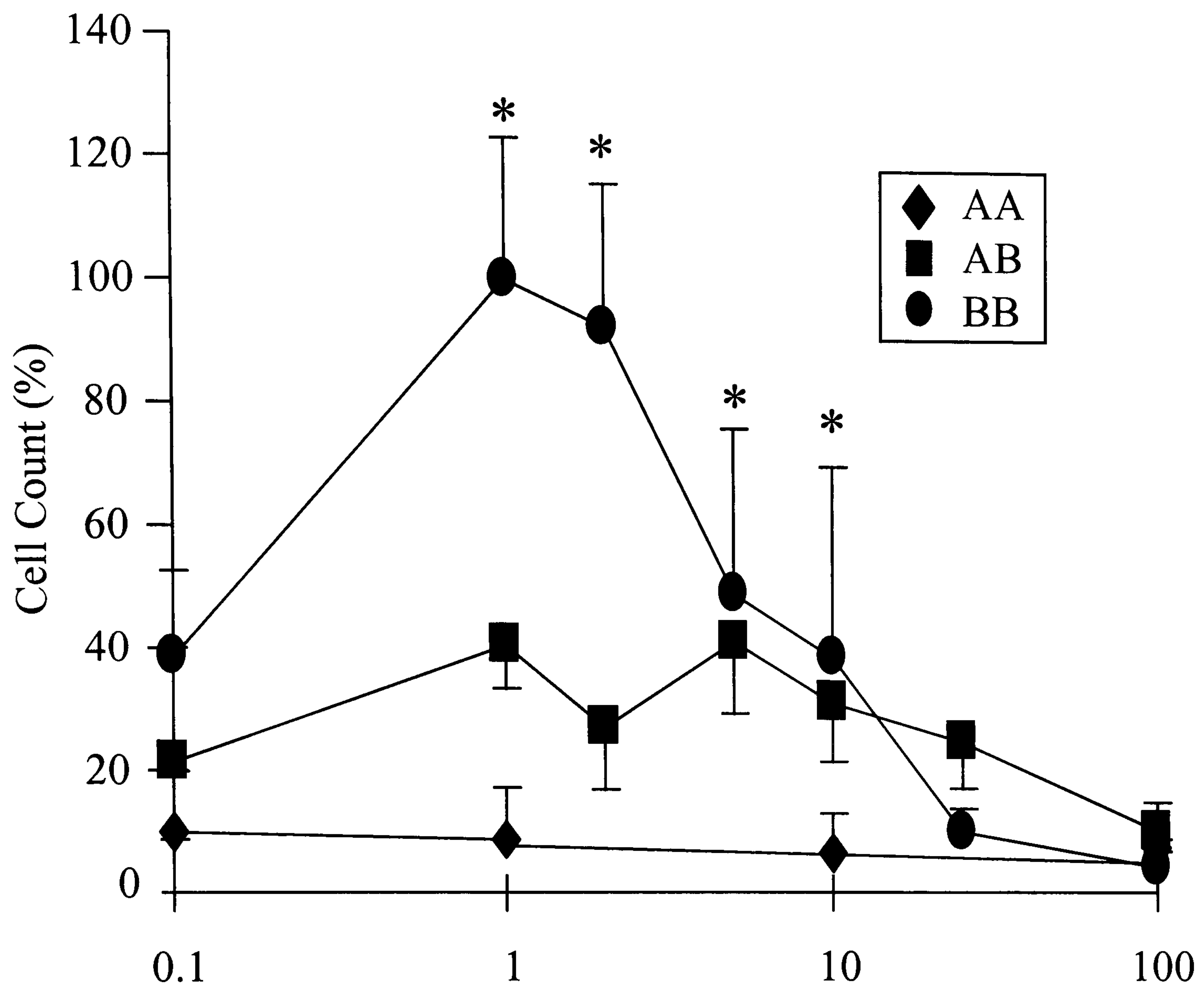


Figure 2. The effect of platelet-derived growth factor (PDGF)-AA, AB and BB on migration in cultured human saphenous vein smooth muscle cells determined using a modified Boyden chamber assay. Data are mean $\pm$ SEM for 4 separate cell lines and are expressed in percentage terms relative to the response to 2 ng/mL PDGF-BB. * denotes $p < 0.05$ by Friedman and Conover.

Figure 3.

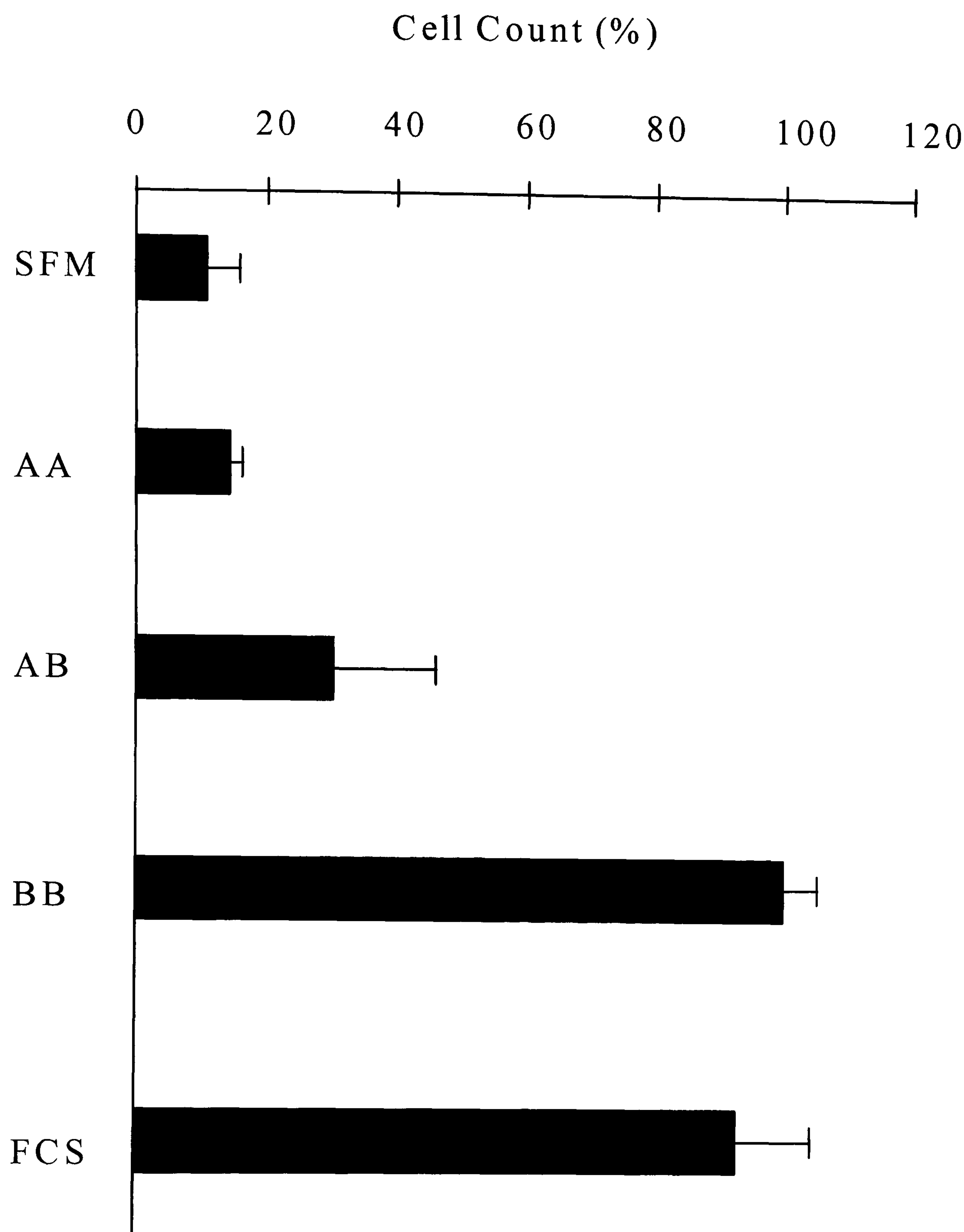
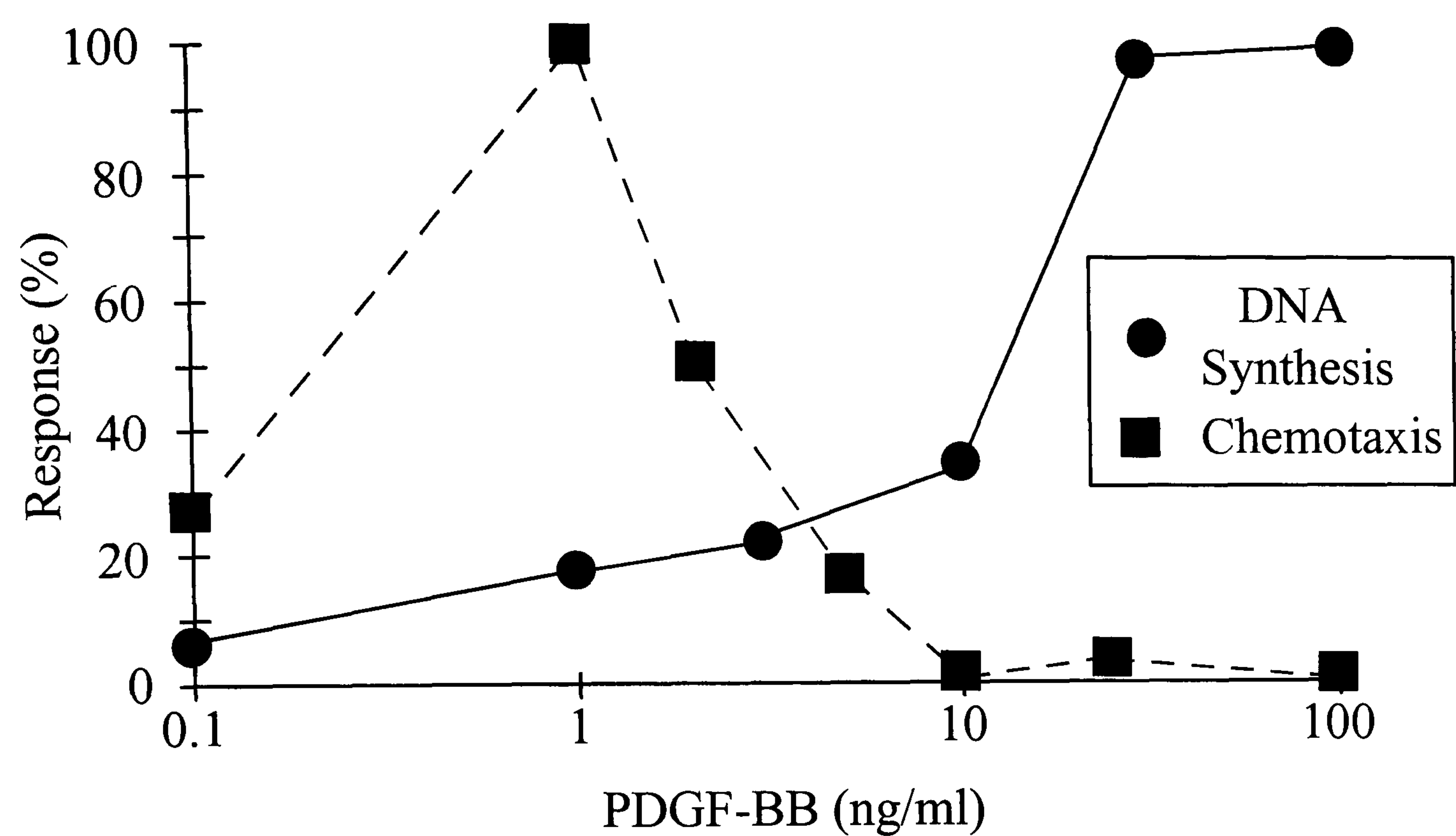


Figure 3. Comparison between the chemotactic responses induced by serum free medium (SFM), foetal calf serum (FCS, 15% vol/vol) and the concentrations of PDGF-AA, PDGF-AB and PDGF-BB which gave the maximal response in a given cell line. Data are mean $\pm$ SEM for 4-11 separate cell lines expressed as a percentage of the mean response to PDGF-BB.

Figure 4.

(a)



(b)

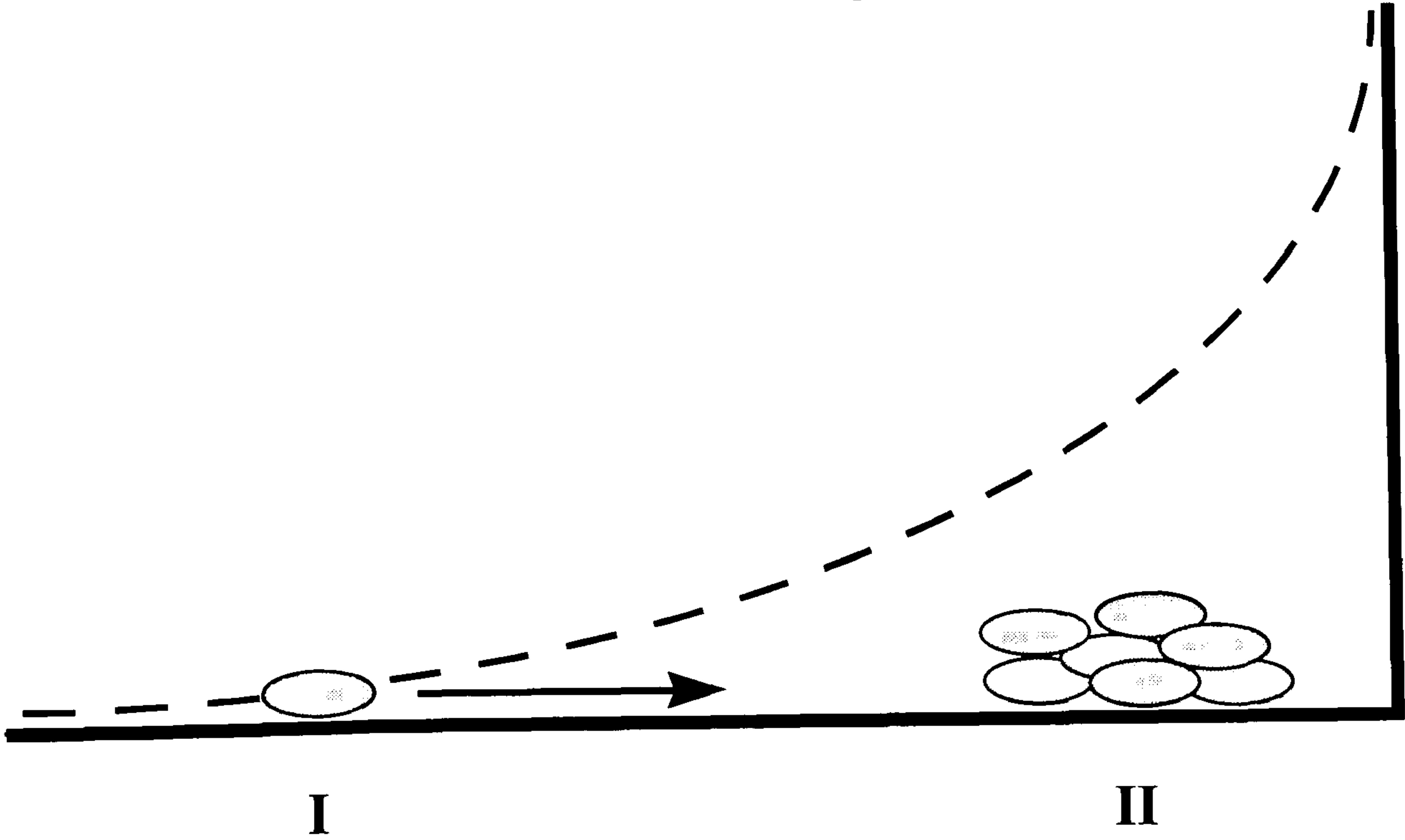


Figure 4. (a) Superimposed plots of the typical chemotaxis and [^3H]-thymidine incorporation dose-response relationships in response to platelet-derived growth factor (PDGF)-BB in human saphenous vein smooth muscle cells. Data are expressed as the percentage of the maximum response for both DNA synthesis and chemotaxis.

(b) Schematic drawing of a model system aligned with the chemotaxis and DNA synthesis profile described in (a). A source of PDGF-BB (located at the vertical bar) gives rise to a concentration gradient of PDGF-BB (dotted line) declining with increasing distance away from the source. A smooth muscle cell (position I) is exposed to a small concentration of PDGF-BB sufficient to induce chemotaxis towards the source (depicted by the arrow). As the cell ascends the concentration gradient the signal for chemotaxis is switched off (refer to (a)), but the signal to proliferate is switched on. The result is the location of proliferating cells at position II.

Figure 5.

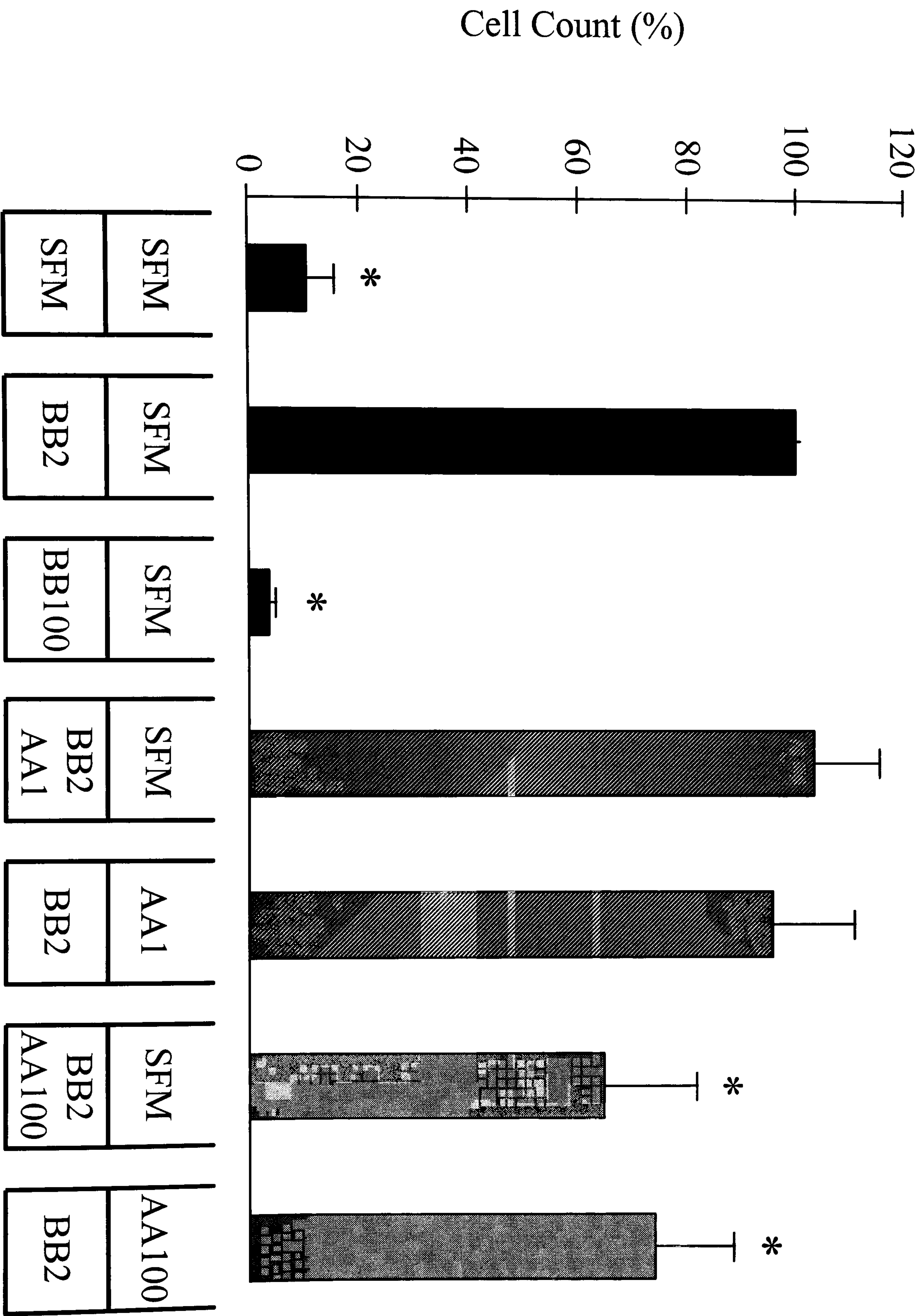


Figure 5. The effect of platelet-derived growth factor (PDGF)-AA on PDGF-BB induced chemotaxis in human saphenous vein smooth muscle cells. SFM, AA and BB denote serum free medium, PDGF-AA and PDGF-BB respectively and the numbers denote the concentrations in ng/mL. The schematic representations of the modified Boyden chambers indicate to which compartment the growth factors were added. Data are mean $\pm$ SEM for 4 separate cell lines expressed as the percentage of the response to PDGF-BB (2 ng/ml) alone. * denotes $p < 0.05$ by Friedman and Conover.

Figure 6.

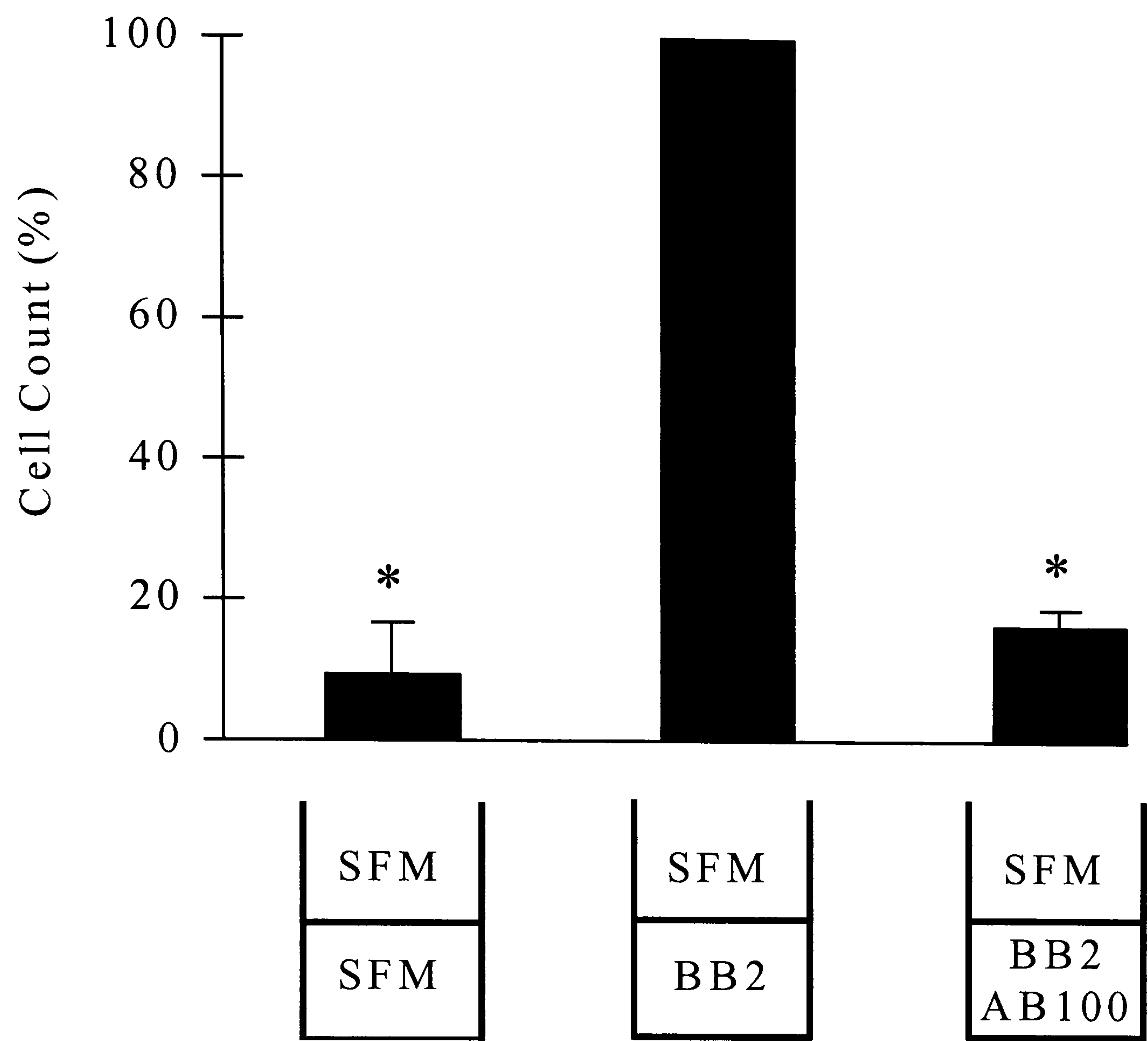


Figure 6. The effect of platelet-derived growth factor (PDGF-AB) on PDGF-BB induced chemotaxis in human saphenous vein smooth muscle cells. SFM, AB and BB denote serum free medium, PDGF-AB and PDGF-BB respectively and the numbers denote the concentrations used in ng/mL. The schematic representations of the modified Boyden chambers indicate to which compartment the growth factors were added. Data are mean $\pm$ SEM for 4 separate cell lines expressed as the percentage of the mean response to PDGF-BB (2 ng/ml) alone. * denotes $p < 0.05$ by Friedman and Conover.

Figure 7.

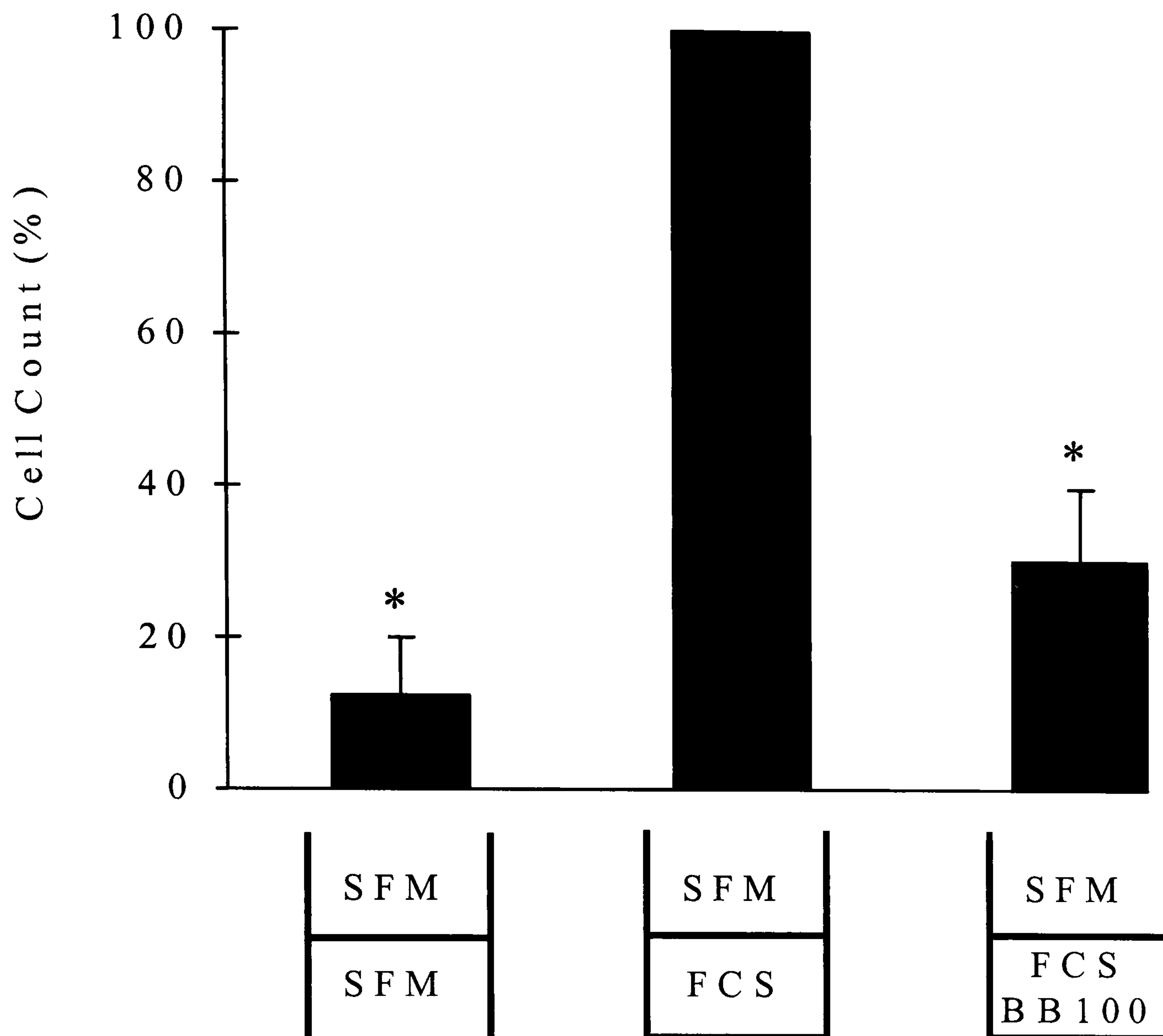


Figure 7. The effect of platelet-derived growth factor (PDGF)-BB on foetal calf serum (FCS, 15% vol/vol) induced chemotaxis in human saphenous vein smooth muscle cells. SFM, and BB100 denote serum free medium and PDGF-BB (100 ng/mL) respectively. The schematic representations of the modified Boyden chambers indicate to which compartment the chemotactic agents were added. Data are mean $\pm$ SEM for 4 separate cell lines expressed as the percentage of the response to FCS alone. * denotes $p < 0.05$ by Friedman and Conover.

SUMMARY, CONCLUSIONS AND FURTHER WORK.

SUMMARY

Restenosis reduces the long term benefit derived from either angioplasty or bypass grafting. Its incidence has been variously reported between 30% and 40% following coronary angioplasty and 20%- 30% following infrainguinal bypass grafting (Popma et al. 1992 Taylor et al. 1990). The process commences immediately following the provoking injury but is not usually clinically apparent until 6 weeks. The lesions continue to mature (laying down extracellular matrix) for up to 18 months. Retrospective studies have demonstrated that early detection and intervention to correct stenotic lesions leads to improved graft patency (Berkowitz et al. 1989, Moody et al. 1990 and Mills et al. 1995)

The pathogenesis of restenosis is multifactorial and the process is collectively known as myointimal hyperplasia (MIH). It appears to result from intimal injury, and medial exposure to platelets. It has three components: (i) migration of a proportion (~ 30%) of medial smooth muscle cells to the intima; (ii) proliferation of these cells; (iii) laying down of extracellular matrix (Dilley et al. 1988, Forrester et al. 1991). The process occurs in all individuals but in the

majority of cases, it is self limiting and has no deleterious effects. However, in approximately 20- 30% it is excessive and causes restenosis which may result in the return of ischaemia and the progression of symptoms.

Attempts to modify MIH in humans by pharmacological interventions have met with very limited success, many agents having been tested during more than a decade of intensive research. Interventions that have shown promise in limiting restenosis in laboratory animal models have not led to similar findings in clinical trials (Ferrell et al. 1992).

Approximately 20 years ago it was found that heparin inhibited vascular smooth muscle proliferation, both *in vivo* and *in vitro* (Clowes et al. 1977, Guyton et al. 1980). When examining the effect of heparin on vascular smooth muscle proliferation, a pronounced heterogeneity of responses has been demonstrated. Of particular interest was the finding that the population of patients with restenoses possessed vein derived cells that were predominantly, resistant to the anti-proliferative effects of heparin. Even more striking was the finding that vascular smooth muscle cells cultured from unaffected vessels in these same individuals shared this tendency to resistance (Chan et al. 1993). Other work has shown no difference in the rate of proliferation of smooth muscle cells taken from various sites in the same vessel, or from different vessels in the same individual. The same is true of cells from successive sub-cultures to the point of senescence (Munro et al. 1994).

A prospective study was, therefore, undertaken (and reported in chapter 4) to correlate cultured VSMC responses to heparin with subsequent graft behaviour. Unused vein segments from 62 patients undergoing infra-inguinal bypass were explanted for VSMC culture. Heparin resistance was assessed by increase in cell number over fourteen days in response to 15% foetal calf serum +/- 100µg/ml heparin. All patients were followed up in a dedicated outpatient clinic for at least one year and their grafts reviewed clinically, with ankle pressures, and three monthly Duplex surveillance. Graft stenoses were confirmed with angiography. After a minimum of one year of follow-up, cultured VSMC associated with non-stenosed grafts are sensitive to heparin's anti-proliferative effects, whereas cultured VSMC associated with stenosed or occluded grafts display resistance to heparin. These prospective data strongly support the hypothesis that VSMC heparin resistance is a marker for excessive MIH and hence graft stenosis.

The glycosaminoglycan heparin inhibits vascular smooth muscle cell (VSMC) proliferation and migration, but the mechanism of its antiproliferative action remains unclear. Heparin has been reported to bind to high affinity cell surface sites on animal VSMC before undergoing receptor mediated endocytosis resulting in signal transduction into the cytoplasm and modulation of genes involved in proliferation. In the study reported in chapter 5, I have characterised the binding of [³H]-heparin to human VSMC and examined whether there is any relationship between the affinity of [³H]-heparin binding and the antiproliferative effect of heparin and its structural analogues.

At 4°C [³H]-heparin binding to human VSMC occurred in a specific, time and concentration-dependent manner. Ligand binding appeared to occur on the cell surface and was both saturable and reversible. Kinetic and steady state data indicated a single class of binding sites. The pharmacology of [³H]-heparin binding was examined in displacement studies using unlabelled heparin or its analogues. Similarly, their antiproliferative effect was determined in DNA synthesis and proliferation assays. The rank potencies of heparin and its structural analogues with regard to the displacement of [³H]-heparin binding and effect in DNA synthesis assays on human VSMC differed and there was a marked difference in estimated affinities in these assays. In summary, I have characterised the heparin binding site on human saphenous vein-derived VSMC. My findings suggest that the antiproliferative action of heparin and its analogues is unlikely to simply reflect an interaction with the cell-associated heparin binding site defined in these studies.

In the sub-group of 15 patients where binding, growth and clinical data (from chapter 4) were known, 8 patients had patent grafts at the end of 1 year while stenoses had been detected in the remainder. Both heparin binding and growth inhibition by heparin were again significantly correlated with clinical outcome. As one would anticipate from the overall patient data there was also a strong correlation between binding and growth inhibition in this sub-population for the three parameters. I have demonstrated for the first time a cellular marker in vascular smooth muscle which is highly predictive of subsequent stenosis in vein graft recipients and have also presented evidence on which to base the formulation of a possible mechanism.

The findings in chapter 5 suggest that the antiproliferative action of heparin and its analogues is unlikely to simply reflect an interaction with the cell-associated heparin binding site. I therefore, examined some of the peptide growth factors (produced by VSMC and endothelial cells) and their interaction with heparin and human VSMC (in chapters 7 and 8) to further elucidate heparin's mechanism of action. Tritiated thymidine incorporation was used to measure DNA synthesis. The results demonstrate all the examined growth factors in isolation result in some degree of VSMC proliferation, some more than others. However, in the vessel wall these growth factors do not act in isolation but will all act in concert and interact with the extracellular matrix. This is rich in heparan sulphate which is structurally similar to heparin. In chapter 8, I have examined the ability of these growth factors to stimulate VSMC proliferation in the presence of heparin. This revealed that heparin's action is not purely related to its ability to bind to growth factors extracellularly but may also be related to an intracellular site of action, possibly in the cell cycle.

From chapter 7 it appears that the PDGF family of growth factors are extremely effective at stimulating DNA synthesis. The effect of the three platelet-derived growth factor (PDGF) isoforms AA, AB and BB on DNA synthesis and chemotaxis on cultured human saphenous vein VSMC was investigated. The AB and BB isoforms stimulated DNA synthesis in a concentration dependent manner as measured by [methyl ^3H]-thymidine incorporation. The maximum effects of PDGF-AB and PDGF-BB were not significantly different and the EC50 values 5 ± 1 ng/mL and 10 ± 1 ng/mL

respectively. The AA isoform elicited no significant incorporation of [methyl ^3H]-thymidine. Chemotaxis, as determined using modified Boyden chambers, yielded efficacies $\text{BB} \gg \text{AB}$, $\text{AA}=0$. For BB the concentration-response relationship displayed a pronounced peak, occurring at 1-10 ng/mL, with no response above this range. The AB isoform (and to a lesser extent the AA isoform) was able to inhibit BB induced chemotaxis. Furthermore, at high concentrations, BB itself was able to prevent chemotaxis induced by foetal calf serum. These results indicate that at low concentrations of PDGF there is little or no signal for DNA synthesis but there is a strong stimulation of chemotaxis. At high concentrations there is a strong positive signal for DNA synthesis but a strong negative signal for chemotaxis.

CONCLUSIONS

CHAPTER 4

- VSMC derived from patients with vein graft stenoses are resistant to growth inhibition by heparin.
- Heparin resistance is a highly significant predictor for the development of subsequent stenosis.
- Conventional risk-factors for atherosclerosis demonstrate little or no consistent association with clinical outcome.
- There is no difference in heparin sensitivity between arteriopaths and non-arteriopaths.

CHAPTER 5

- The binding of [^3H]-heparin to human VSMC occurs through a single class of sites in a specific, saturable, reversible, time and concentration-dependent manner.

CHAPTER 6

- There is a clear relationship between heparin resistance and heparin binding.

- In a sub-group of patients in whom I have data on clinical outcome, heparin inhibition and heparin binding a strong correlation has been confirmed for these parameters.

CHAPTER 7

- PDGF-AB, PDGF-BB, EGF and FGF are all effective stimuli to DNA synthesis, although less effective this is not significantly different from FCS.
- IGF (with and without insulin in the serum free medium) and PDGF-AA are both significantly weaker mitogens for the VSMC than FCS.

CHAPTER 8

- Heparin was consistently unable to prevent VSMC DNA synthesis when stimulated by: PDGF-AB, PDGF-BB and EGF.
- Heparin consistently inhibited VSMC DNA synthesis when stimulated by: FGF, IGF, PDGF-AA and FCS.

CHAPTER 9

- PDGF-BB induced a large chemotactic response, PDGF-AB a weak response and PDGF-AA no response.
- The peak response to PDGF-BB was not significantly different from the response to 15% FCS.

- The form of the PDGF-BB chemotactic concentration-response relationship, was markedly different from that of the proliferative response and the concentrations required to stimulate the two effects differed by between one and two orders of magnitude. This observation supplies evidence that the signalling pathways involved in PDGF induced growth and migration are divergent.

FURTHER WORK

The role of extracellular matrix proteins as regulators of cell growth and differentiation is a possible mechanism which needs further investigation. Altered responsiveness to heparin may itself be a marker for a more fundamental disturbance of growth regulation in the vascular wall, though this does not preclude the possibility that a failure to interact appropriately with glycosaminoglycans in the vessel wall has in itself an important role in the disease process. Understanding these mechanisms and how they could be modulated will contribute to better understanding of the biology of the vascular wall and may also lead to more rational and effective management of an important clinical problem, with the intriguing prospect of identifying individuals at increased risk of graft failure and ultimately reducing the likelihood of an unfavourable outcome.

The assays used in this work have relied on cultured human VSMC and in order to culture cells and perform assays on them, three to four months is required. The acceleration of determining heparin resistance would allow targeting of patients at high risk of stenosis for aggressive surveillance or even therapy to prevent stenoses. Leung and colleagues (1989) demonstrated heparin binding to the surface of human monocytes and so blood would seem the next obvious object of investigation.

I have demonstrated the complexity of responses of human VSMC to the three isoforms of PDGF in terms of their ability to stimulate DNA synthesis and

chemotaxis. However, the role of heparin in this model has not been tested and needs to be examined. Also the growth factor assays were all performed in isolation, it is not known what the effect of these growth factors is on human VSMC when added to the cells in combination.

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