Application of Proteomics to Analysis of Chondrocyte Gene Expression

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Abstract

In this project, 1 and 2 dimensional-electrophoresis and mass spectrometry were used to study differential protein expression in chondrocytes and cartilage explants in response to cytokine stimulation and arthritic disease.

Seven proteins regulated by IL-1 in monolayer chondrocytes were identified by 1D-electrophoresis and mass spectrometry. These were manganese superoxide dismutase, heparin-binding glycoprotein-38, collagenase (MMP-1), stromelysin (MMP-3), serum amyloid A and complement component C3. Comparison of proteins secreted by monolayer cells and cartilage explants showed that constitutive protein expression varied with respect to the physiological surroundings of the cells, whereas proteins induced in response to IL-1 did not.

Proteoglycan aggregates are a major component of cartilage. The glycosaminoglycan (GAG) side chains of proteoglycans are polyanionic and bind to proteins at low ionic strength and hinder their analysis by isoelectric focusing. To study secreted proteins from cartilage explant medium, a novel method was developed to remove proteoglycans by precipitation with cetylpyridinium chloride (CPC), which provided reproducible 2D pattern.

The technique was used to compare protein expression in normal and osteoarthritic (OA) human cartilage. 179 protein spots excised from the stained gels were identified by mass spectrometry and represent the products of 92 distinct genes. The 2D pattern of metabolically labelled secreted proteins suggested the existence of a stable and consistent disease phenotype. This was characterised by elevated levels of collagen type II and collagen type II fragments, together with enzymes involved in collagen synthesis and processing. Increased protein synthesis of collagen type II is in agreement with reports of increased mRNA for the protein in OA cartilage.

Several growth factor-like molecules were detected in OA cartilage, including cytokine-like protein C-17 and connective tissue factor. In contrast to normal controls, OA cartilage produced large amounts of the βA chain of activin/inhibin, a member of the TGFβ super family, which was present both as the fully processed product and as at least 6 glycoforms of the precursor protein. I hypothesise that activin plays a crucial

role in orchestrating the pathological changes observed in OA cartilage, and this possibility is now being investigated.

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Abbreviations

1D-	One dimensional
2D-	Two dimensional
$ACTH_{clip18-39}$	Adrenocorticotropic hormone fragment 18-39
ADAM TS	A disintegrin and metalloproteinase with thrombospondin motif
APS	Ammonium persulphate
BMP	Bone morphogenic protein
bFGF	Basic Fibroblast growth factor
CEP-68	Chondrocyte expressed protein-68
CM	Medium conditioned by cartilage
CID	Collision-induced dissociation
CILP	Cartilage-intermediate layer protein
COMP	Cartilage oligomeric matrix protein
CPC	Cetyl pyridinium chloride
CS	Chondroitin sulphate
CTGF	Connective tissue growth factor
DEAE	Diethylaminoethyl
DMB	Dimethyl methylene blue
DMEM	Dulbecco's modified Eagle's medium
DS	Dermatan sulphate
DTT	Dithiothreitol
EBNA	Episomal mammalian expression vector system
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellulary regulated kinase
ESI	Electrospray ionization
EST	Expressed sequence tag
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FN	Fibronectin
FN-fs	Fibronectin fragments
FSH	Follicle-stimulating hormone
GAG	Glycosaminoglycan
gp38	Heparin-binding glycoprotein 38 (porcine)
gp-39	Heparin-binding glycoprotein 39 (human)/YKL-40
HS	Heparan sulphate
IGD	Interglobular domain
IGF	Insulin-like growth factor
IL-1	Interleukin-1
IL-17	Interleukin-17
IRAP	Interleukin-1 receptor antagonist protein

IAA	Iodoacetamide
IPG	Immobilised polyacrylamide gel
ITS	Insulin, transferrin, sodium selenite
MALDI	Matrix assisted light desorption ionisation
MAP	Mitogen activated protein
MMP	Matrix metalloproteinase
MnSOD	Manganese superoxide dismutase
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MT-MMP	Membrane bound MMP
m/z	Mass to charge ratio
NCBI	National centre for biotechnology information
NO	Nitric oxide
n.r.	Non-redundant
OA	Osteoarthritis/osteoarthritic
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PMSF	Phenyl methyl sulphonyl fluoride
PVDF	Polyvinylidene difluoride
Q-Tof	Quadrapole-Time of flight
RA	Rheumatoid arthritis/arthritic
RT	Run-through
SAA	Serum amyloid A protein
SPARC	Secreted protein acidic and rich in cysteine
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TGF β	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinase
TNF α	Tumour necrosis factor α
TSG-6	TNF-stimulated gene-6
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1. Background

This thesis describes the use of two-dimensional (2D)-electrophoresis and mass spectrometry (MS) to analyse protein expression in articular cartilage. My goals have been to develop a 2D-map of proteins secreted by chondrocytes, and to use this to study regulation of protein synthesis by cytokines and other factors. As will be shown, there are both advantages and disadvantages in using 2D- compared to one-dimensional (1D)-electrophoresis.

The techniques were also used to study how proteins secreted by chondrocytes differed with respect to their physiological surroundings. The biosynthetic patterns of proteins secreted by chondrocytes in monolayer culture and in explants were compared.

Much of the work was done with porcine cartilage because animal tissue is relatively easier to obtain than human. In chapter 5, the techniques were applied to human cartilage to compare proteins secreted from normal and osteoarthritic (OA) cartilage. The result shows it is possible to use the approach to investigate disturbances of protein expression in disease. It could be useful for defining phenotypic changes in the progression of osteoarthitis, and for evaluating chondrocyte behavior in attempts at tissue engineering. Several proteins were found that differed in expression between normal and OA cartilage.

In this chapter, I will first give an introduction to cartilage structure and biology. The morphological and biochemical changes that occur in cartilage in disease will be explained together with an overview of osteoarthritis. Techniques and equipment used for protein identification will then be introduced. The advantages of using cartilage in this approach will be discussed and an overview of the function and use of MS will be given.

1.1 Structure and function of articular cartilage

The major structural polymers of cartilage are type II collagen and proteoglycan aggregates, composed of aggrecan, link protein and hyaluronic acid. The

highly hydrophilic aggrecan aggregate is embedded in the collagen fibrillar network (Comper, 1991). It is underhydrated and its swelling pressure gives cartilage its ability to resist compression. The collagen network gives cartilage tensile strength and is aligned in a way to help distribute the forces associated with compression.

The only cells in cartilage, the chondrocytes, contribute less than 5% by volume (Poole, 1986) and are responsible for normal synthesis and degradation of matrix components. In normal cartilage the cells have low metabolic activity, low matrix turnover and divide rarely.

Cartilage consists of 65-80% water, 15-22% collagen (wet weight) and 4-7% aggrecan (wet weight)(Comper, 1991).

Since the chondrocytes are sparsely distributed in the cartilage, they have little direct cell to cell contact and interact only with the extracellular matrix and with molecules diffusing through the matrix. Cartilage has neither blood supply nor contact with the nervous system (Tortora and Grabowski, 1994) and obtains nutrients by diffusion through the matrix. Articular cartilage has a very limited capacity to repair itself either in response to mechanical injury or after inflammatory damage as in rheumatoid arthritis (RA) (described in 1.2.1) (Crawford and Campbell, 1969).

1.1.1. Morphology of articular cartilage

The structure of cartilage is highly ordered and varies with respect to the distance from the articular surface. Four zones or layers can be identified within the cartilage: the superficial zone, the transitional zone, the radial zone and the zone of calcified cartilage (Buckwalter and Hunziker, 2000). The different zones vary in water, proteoglycan and collagen concentrations and in the size of the aggregates. The shape, size and orientation of the cells vary between the different zones (Aydelotte et al., 1992) as well as the concentration and metabolic activity of the cells (Wong et al., 1996).

In the superficial zone the cells are flattened and aligned parallel to the surface. The thin collagen fibrils generally run parallel to each other and to the articular surface. They maintain the tensile properties of the cartilage so that it can

resist shear forces during joint use (Buckwalter and Hunziker, 2000). The cells in this zone produce more collagen and less proteoglycan compared to the other zones (Aydelotte et al., 1992; Wong et al., 1996). Also the water content is highest in this zone.

The transitional zone has a morphology and matrix composition that is intermediate between the superficial and radial zones. The cells are more rounded and produce more proteoglycan and less collagen (although the fibrils are thicker) than the superficial zone. The concentration of water is also lower.

In the radial zone, which is the largest, the spherical cells are aligned in columns perpendicular to the joint surface. The collagen fibers are thickest here, the proteoglycans most concentrated and the water content lowest.

On the boundary between the radial and calcified cartilage zones is the 'tidemark', seen as a thin line under the microscope. The purpose and structure of this area is uncertain (Oegema and Thompson, 1992). It may result from concentration of basophilic calcified material at the interface between calcified and uncalcified matrix and thus represent a "high water mark" for calcification.

The thin layer of calcified cartilage separates the cartilage from the subchondral bone. The cells here are almost entirely surrounded by calcified cartilage suggesting that they have very low metabolic activity.

1.1.1.1 Cellular activity in articular cartilage

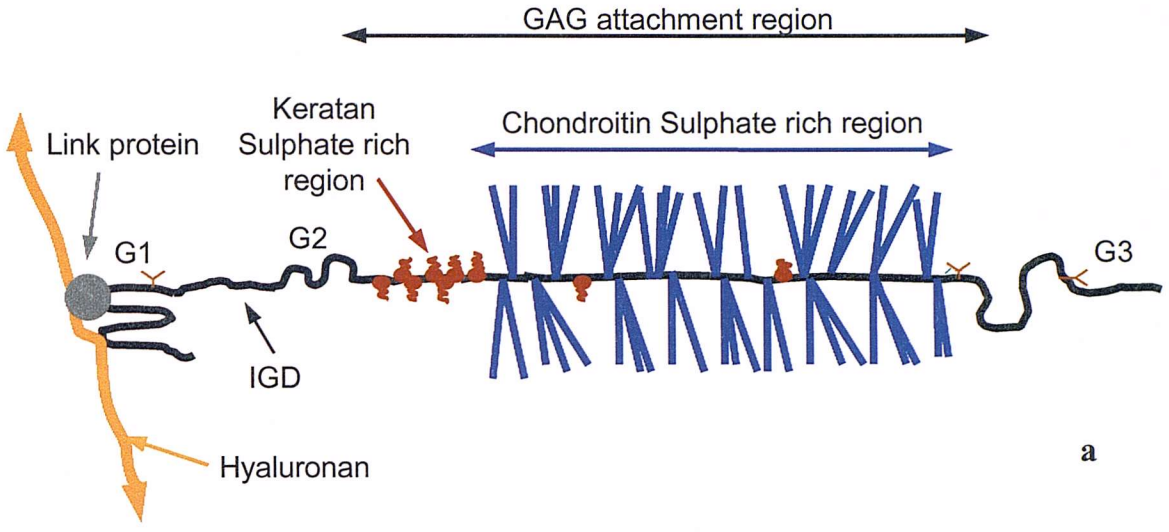
In normal cartilage the cells rarely divide and are responsible for slow matrix turnover. Synovial fluid has a low oxygen tension (4-10 %) (Ferrel and Najafipour, 1992) and cartilage can be even more hypoxic. Articular cartilage has been suggested to have an oxygen tension of about 10 % at the articular surface and 1 % in the deepest layer (Silver, 1975). Therefore, chondrocyte metabolism is mainly anaerobic with the conversion of glucose to lactic acid (reviewed in Lee et al, 2002).

Cartilage has low metabolic activity compared with tissues like muscle or bone (Buckwalter and Hunziker, 2000), when cartilage has been damaged, cellular activity increases and the cells form clusters (see 1.2.2.2 and 1.2.2.4)

The metabolic activity and morphology of the chondrocytes are affected by mechanical loading of the joint. Normal cartilage in load-bearing areas are thicker and the cells are larger compared to in non-load-bearing cartilage. The proteoglycan concentration is higher in load-bearing cartilage (reviewed in Urban, 1994). However, under high hydrostatic-pressure of articular cartilage, proteoglycan synthesis can be inhibited (Lammi et al., 1994).

1.1.2 Proteoglycans

Proteoglycans are large molecules that consist of a protein backbone to which glycosaminoglycan (GAG) side chains and *N*- and/or *O*-linked oligosaccharides are covalently attached. GAGs are a family of linear polymers composed of repeating disaccharide units (fig 1.1) usually about eighty units long for large proteoglycans. One of the two monosaccharides is always either *N*-acetylglucosamine or *N*-acetylgalactosamine. One or more of the hydroxyls of the aminosugar are esterified with sulphate. The second sugar is usually an uronic acid (glucuronic or iduronic). The combination of the sulphate groups and the carboxylate groups of the uronic acid residues makes the GAGs highly anionic. Large proteoglycans can contain as much as 95 % carbohydrate by weight (Alberts et al, 1994).



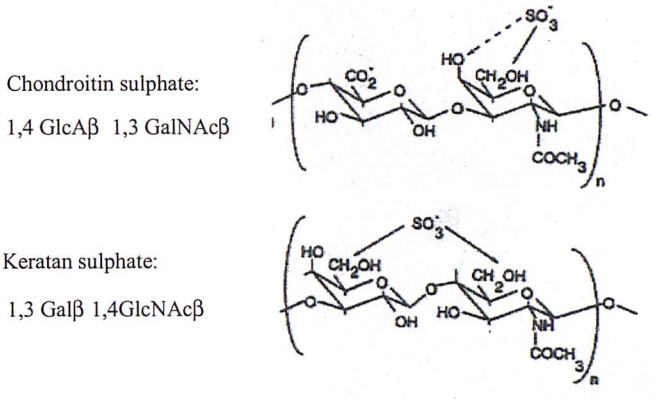


Fig 1.1. A schematic view of the structure of the major proteoglycan found in cartilage, aggrecan (a), adapted from Heinegård and Oldberg, 1989. The disaccharide units of two primary GAG chains in cartilage (b).

Aggrecan is the major proteoglycan in articular cartilage, and comprises approximately 90 % of the total proteoglycan. Up to 100 chondroitin sulphate (CS) and keratan sulphate chains are covalently attached to the protein core of aggrecan (220-250 kDa) (Poole, 1986) (fig 1.1). Chondroitinase ABC lyase cleaves dermatan sulphate (DS) and 4- and 6-sulphated CS chains only and can be used to digest the GAG chains to study the type of proteoglycan or proteoglycan fragments. The number of sulphate groups on the GAG-chains can also be used to estimate the quantity of proteoglycan in cartilage.

The aggrecan protein core is organised into three distinct globular domains (G1, G2 and G3). The *N*-terminal G1 and G2 are separated by an interglobular domain (IGD), which is sensitive to proteolytic cleavage. G2 is separated from the *C*-terminal G3 domain by a long GAG attachment region (fig 1.1).

200 or more aggrecan molecules can bind to hyaluronan via the G1 domain. The interaction is stabilised by link protein (40 kDa) and the size of the aggregate can be 100 million daltons or above.

Small CS/ DS proteoglycans in articular cartilage are biglycan (core, 38 kDa) and decorin (core, 42 kDa). Decorin has one GAG chain and biglycan has two (Hardingham and Fosang, 1992). Furthermore, two small keratan sulphate proteoglycans are found in articular cartilage, fibromodulin (core, 43 kDa) and lumican (core, 39 kDa).

Decorin and fibromodulin bind collagen I and II via their protein cores and may play a role in maintaining the organisation of the extracellular matrix and regulating the thickness of collagen fibrils in the tissue (Scott and Haigh, 1988). Decorin and biglycan also bind TGFβ (Hildebrand et al., 1994; Yamaguchi et al., 1990). Biglycan does not bind collagen and its function in cartilage is uncertain. Fibromodulin contains GAG chains only in the foetus and juvenile, and is present as a glycoprotein in the adult (Roughley et al., 1996). Lumican is present predominantly as a glycoprotein in the adult and is absent in the foetus and juvenile (Grover et al., 1995). Like the other small proteoglycans, fibromodulin and lumican interact with the fibrillar collagens in the matrix.

Perlecan is a large CS/heparan sulphate (HS) proteoglycan (core, 470 kDa) containing three GAG chains (Iozzo et al., 1994). It is a specific component of basement membranes. Its presence in cartilage is surprising since the tissue lacks basement membranes (SundarRaj et al., 1995). Mice with a null mutation in the perlecan gene lost the integrity of basement membranes and also developed defects in their cartilage with an achondroplastic phenotype (Costell et al., 1999). A reduced number and shortening of collagen fibrils was detected, suggesting a role for perlecan in maintaining the collagen network. Furthermore, perlecan binds basic fibroblast growth factor (bFGF) through its HS chains and could serve as its co-receptor, thus playing a role in bFGF mediated pathways (Aviezer et al., 1994).

Chondrocytes also express the cell surface proteoglycan syndecan. This proteoglycan carries one or two HS chains, alone or in combination with CS chains (Mali et al., 1990). The function of syndecan is uncertain but it could act as a signal transducer by binding various effector molecules via its HS chains (Hardingham and Fosang, 1992).

1.1.3 Collagen

Collagens are a family of insoluble fibrous proteins found extensively in connective tissues. Collagen type II is the major protein in cartilage, which also contains small amounts of types VI, IX, X and XI.

Type II and XI are fibrillar collagens. Their rope-like triple helical structure is formed from three polypeptide chains, called α-chains and stabilised through hydrogen bonds. The α-chains are constructed of a series of Gly-Pro-X triplet motifs, where X usually is hydroxyproline (OH-P), and more seldom lysine or hydroxylysine. They are synthesised as pro-α chains with additional propeptides at both the C- and *N*-terminal ends. After formation of the triple helices, the procollagen molecule is secreted into the extracellular space and the propeptides are removed by specific proteolytic enzymes. The collagen molecules then assemble into larger collagen fibrils stabilised by covalent intramolecular and intermolecular cross-links. These fibrils can have a diameter of 30 to 200nm (Comper, 1991).

Type XI collagen exists within the collagen type II fibrils, where it regulates the formation of the fibril and mediates interactions between collagen and proteoglycans.

The winding of the fibrillar collagen chains is such that the peptide bonds are buried in the interior of the structure. Thus, triple-helical collagen is very resistant to proteases. The only group of enzymes that can efficiently attack the triple helical collagen structure are the collagenases (Tyler, 1991) (see 1.1.5.1 Matrix metalloproteinases (MMPs)).

The smaller type VI, X and IX collagens belong to the fibril–associated collagens. Their triple helical structure is interrupted by one or two short nonhelical domains that make them more flexible than fibrillar collagens. The non-helical domains often have a globular structure.

In type IX collagen, the propeptides are not cleaved and it does not aggregate to form fibrils. Instead it binds covalently to the surface of collagen type II, where it is thought to mediate interaction of collagens fibrils with one another and with other matrix molecules.

Collagen type X has a very limited tissue distribution and is synthesised by hypertrophic chondrocytes in cartilage undergoing endochondral ossification and in the hypertrophic zone of epiphyseal cartilage were it may aid calcification. It is also present in the calcified zone of adult articular cartilage.

Type VI collagen has a very short helical domain with large non-collagenous domains at each end. It is glycosylated and present in most tissues (reviewed in Timpl and Engel, 1987). It binds to type II collagen, decorin and to hyaluronan among others. It has been suggested that it participates in organising the pericellular matrix and the attachment of chondrocytes to the extracellular matrix.

1.1.4 Other proteins in cartilage

One of the most abundant non-collagenous proteins is cartilage oligomeric matrix protein (COMP) (Hedbom et al., 1992). This acidic glycoprotein belongs to the thrombospondin family and is also known as thrombospondin-5. It consists of five

100 kDa disulphide bonded subunits and binds collagens type I and II, suggesting involvement in collagen fibril organisation (Rosenberg et al., 1998). COMP is also found in the cartilage growth plate and may play a role in regulation of cell growth and proliferation (Shen et al., 1995).

Cartilage-intermediate-layer protein (CILP) is a 92 kDa protein, abundant in the mid-zone of cartilage but not found in the superficial or deeper zones of cartilage. It seems to be specific to cartilage, but its function is unknown (Lorenzo et al., 1998a; Lorenzo et al., 1998b).

Fibronectin (FN) is an extracellular adhesive glycoprotein that helps cells attach to the matrix. It consists of two subunits of approximately 220 kDa, joined by disulphide bonds near the *C*-terminal forming a dimer. Each subunit is folded into a series of functionally diverse rodlike domains separated by regions of flexible polypeptide chain. FN is easily degraded into fragments (FN-fs). Cleavage by proteinases such as thrombin and cathepsin D produce 29 kDa, 50 kDa, 110-140 kDa, 35 kDa and 25 kDa fragments. In patients with osteoarthritis (OA) (described in 1.2.2) or RA, elevated levels of intact FN and fragments have been detected in cartilage and synovial fluid (reviewed in (Homandberg, 1999).

High concentrations of FN-fs (>100 nM) have been reported to have catabolic effects in cartilage whereas lower concentrations (1 nM) are anabolic (Homandberg and Hui, 1994). The most active is the 29 kDa *C*-terminal fragment.

Bovine cartilage stimulated with 1 μM of the 29 kDa or 50 kDa fragments showed an increased release of proteoglycans and increased collagenolyitc and gelatinolytic proteinase release (measured by synthetic substrate cleavage) (Homandberg et al., 1992). Intact FN had very low activity. FN-fs may contribute to protease-mediated damage of cartilage, by inducing release of inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α) (Homandberg et al., 1997) that could be responsible for the effect seen on proteoglycan degradation and proteinase release.

1.1.4.1 Growth factors in cartilage

The metabolism of articular cartilage is regulated by growth factors originating from the cartilage and the synovial tissue and fluid.

Transforming growth factor β (TGFβ) belongs to the TGFβ-superfamily and regulates cell growth and differentiation in connective tissues. It is synthesised by chondrocytes among other cell types (Villiger and Lotz, 1992), and may protect cartilage from damage as it has been shown to enhance matrix production and modulate chondrocyte proliferation (Pujol et al., 1994). Furthermore, it can oppose the effect of IL-1 on articular chondrocytes, reducing the inhibition of proteoglycan and collagen type II synthesis and inhibiting matrix metalloproteinases production (Lum et al., 1996). It can also downregulate the expression of IL-1 receptors in articular chondrocytes (Redini et al., 1993).

TGFβ signals by binding to the TGFβ receptor II (TGFβR II) that subsequently forms a complex with TGFβ receptor I (TGFβRI) that transmits the signal (Wrana et al., 1994). Changes in expression of TGFβ receptors on the chondrocyte surface could lead to a decrease in metabolic activity of the cells and failure to counter the degrading effects on cartilage by IL-1. In a rabbit model of OA, the mRNA level of TGFβRII was considerably decreased in cartilage as the disease progressed (Boumediene et al., 1998). Therefore chondrocytes may lose their sensitivity to TGFβ during the development of OA, which may partially explain the poor ability of cartilage to repair itself. This idea is supported by the work of Serra et al (1997), who generated transgenic mice that expressed a truncated defective TGFβRII. These mice developed a degenerative joint disease resembling OA in humans.

Bone morphogenic proteins (BMPs) also belong to the TGFβ superfamily. They were originally identified as inducers of bone and cartilage formation in ectopic sites in vivo (Reddi, 1992). BMPs have also been shown to play important roles in embryogenesis (Hogan, 1996) and development and maintenance of other tissues (Dudley et al., 1995; Song et al., 1995). At least 20 members have been described.

The most potent in bone and cartilage formation are BMP-2, BMP-4, BMP-6 and BMP-7.

BMPs are present within articular cartilage and play a role in its maintenance. BMP-2 and BMP-7 stimulate the synthesis of aggrecan and collagen by human articular chondrocytes in vitro (Flechtenmacher et al., 1996; Luyten et al., 1992). In contrast to TGFβ, BMP-2 failed to oppose the effect of IL-1 in murine cartilage on proteoglycan synthesis and degradation (Glansbeek et al., 1997). BMP-7, also known as osteogenin-1, has been shown to induce differentiation of chondrocytes and increase proteoglycan synthesis in porcine cartilage explants (Lietman et al., 1997). BMP-7 can oppose the effect of low doses of IL-1β on human articular chondrocytes (Huch et al., 1997). Furthermore, BMPs have been shown to promote cartilage differentiation and protect engineered articular cartilage from destruction (Kaps et al., 2002). Implanted recombinant BMP-2 has been shown to stimulate repair of damaged rabbit articular cartilage, suggesting it could be useful for regeneration of cartilage (Suzuki et al., 2002).

Another group of related molecules are the cartilage derived morphogenetic proteins 1 and 2 that are predominately expressed in bone during embryonic development (Chang et al., 1994). They are present in articular cartilage and may stimulate metabolic activity of chondrocytes (Erlacher et al., 1998).

Connective tissue growth factor (CTGF) is expressed by many cell types and is induced by TGFβ (Moussad and Brigstock, 2000). In chondrocytes it promotes proliferation and differentiation (Nakanishi et al., 2000). It has been reported to be present in the hypertrophic zone of the growth plate (Nakanishi et al., 1997).

Insulin-like growth factor-1 (IGF-1) is a major homeostatic factor, controlling matrix synthesis and degradation (Froesch et al., 1985). In articular cartilage, the message for IGF-1 increased 4 weeks after creation of full-thickness defects (Fortier et al., 2001). Following punch biopsy of rabbit ear cartilage, maximal staining of IGF-1 protein was detected after 7 days (Bos et al., 2001). IGF-1 has not been detected by immunochemistry of normal mature mouse cartilage (Chambers et al., 1997).

Furthermore, it is an inducer of proteoglycan synthesis in normal but not in RA cartilage, suggesting that the arthritic chondrocytes have lost their response to this growth factor (Schalkwijk et al., 1989). Reduced levels of IGF and increased levels of IGF binding proteins have been detected in patients with RA. This may result in reduced levels of free IGFs that can bind to the IGF receptors (Neidel, 2001). Patients with OA also produce elevated levels of IGF binding proteins, thereby blocking IGF receptor activation (Dore et al., 1994).

Vascular endothelial growth factor (VEGF) is important for blood vessel formation (Klagsbrun and D'Amore, 1996). It is a key regulator of angiogenesis in endochondral ossification where vascular invasion of cartilage is necessary for bone formation (Gerber et al., 1999). VEGF mRNA and protein is expressed by the chondrocytes in the foetal growth plate (Garcia-Ramirez et al., 2000). It has also been detected in human articular cartilage (Pfander et al., 2001).

Basic fibroblast growth factor was initially identified as cartilage derived growth factor from foetal bovine scapula cartilage that could stimulate DNA synthesis and cell division of bovine chondrocytes (Klagsburn et al., 1977). It was later found to be identical to bFGF (Lobb et al., 1986), which is a potent angiogenic factor. It has been shown to stimulate protein production in bovine articular cartilage. Low doses of bFGF (3 ng/ml) enhance [^{35}S] proteoglycan release while high doses (30-300 ng/ml) have no effect (Sah et al., 1994). Administration of bFGF to defects in a rabbit articular cartilage promoted differentiation of cells and matrix synthesis (Fujimoto et al., 1999). Our group has shown that bFGF is released upon mechanical injury to cartilage and induces production of matrix metalloproteinases (Vincent et al., 2002).

Chondromodulin-1 is a heparin binding glycoprotein that was purified from foetal bovine cartilage and shown to stimulate growth of cultured chondrocytes in the presence of bFGF (Hiraki et al., 1991). It was later found to be present in the avascular zone of cartilage in developing bone but not in the calcified cartilage (Hiraki et al., 1997). This suggested it could play a regulatory role in blood vessel formation during endochondral bone formation. A low level of chondromodulin-1 mRNA has been detected in adult bovine articular cartilage (Azizan et al., 2000).

Two related proteins, chondromodulin II and III were identified from foetal bovine cartilage, with a lower affinity for heparin than chondromodulin-1 (Hiraki et al., 1996).

1.1.5 Matrix turnover in cartilage

The degradation of extracellular matrix is tightly controlled. Various members of the four classes of mammalian proteinases have been implicated in the turnover of cartilage matrix. Proteinases are classified by the nature of their active site. Cysteine and aspartate proteinases cleave proteins at acidic pH. Serine and metalloproteinases act at neutral pH. The pH of the extracellular matrix is close to neutrality, 6.95-7.4 (Silver, 1975) suggesting a more important role for the neutral proteinases in extracellular proteolysis. Most attention has been given to the matrix metalloproteinases (MMPs) which are secreted or bound to the cell membrane and are suggested to mediate matrix degradation and subsequent joint destruction in RA and OA.

Serine proteinases are released from granules of activated neutrophil granulocytes and are implicated in inflammatory tissue destruction (Del Rosso et al., 1999). Cells can also phagosetose matrix, in which case hydrolysis occurs in the acidic lysosomal/endosomal compartment by cysteine and aspartate proteinases.

1.1.5.1 Matrix metalloproteinases (MMPs)

The matrix metalloproteinases are a large group of enzymes that collectively can degrade all components of the extracellular matrix. More than 20 members have so far been identified. They can be divided into four groups: collagenases, stromelysins, gelatinases and membrane-type metalloproteinases (MT-MMPs).

All have a zinc ion in their active site, which is crucial for the catalytic mechanism. The presence of calcium ions are required for the stability and expression of their activity (Nagase and Woessner, 1999). All MMPs are synthesised as prepro-enzymes and most are secreted as inactive pro-enzymes. Activation of the pro-

enzymes occur generally by cleavage of the propeptide by proteinases and other MMPs. A cysteine residue within a conserved region in the propeptide (the cysteine switch) is ligated to the zinc ion to maintain the enzyme in an inactive form. Some MMPs (MMP-11 and MT1-MMP) are activated intracellulary by a furin-dependent cleavage of the propeptide.

The collagenases comprise at least three human members: MMP-1, MMP-8 and MMP-13. These enzymes cleave the triple helix of collagen types I, II and III at a single site producing ¼ and ¾ fragments. This allows unwinding and further degradation by other proteolytic enzymes.

Stromelysins (MMP-3, MMP-10, MMP-11) have a broad substrate specificity. MMP-3 (stromelysin 1) can cleave aggrecan, link protein, fibronectin and collagen (after initial cleavage by the collagenases). It can also activate proMMP-3 and other MMPs like proMMP-1, proMMP-8, proMMP-9 and proMMP-13 (reviewed in (Nagase, 1998).

Gelatinase A and, B also known as MMP-2 and MMP-9, cleave denatured collagen (gelatin), type IV collagen and laminin.

The membrane-bound metalloproteinases are the most recently discovered group. They differ from the other MMPs by being anchored to the cell surface. Four members have been identified: MT1-MMP, MT2-MMP, MT3-MMP and MT4-MMP. The MT1-MMP cleaves triple helical collagen and has been shown to be involved in activation of proMMP-2 (Sato et al., 1994).

Beside their role in pathological matrix degradation, MMPs are also involved in normal processes like wound healing and development. In disease, MMPs have been associated with arthritic diseases, atherosclerosis, tumour growth and metastasis reviewed in (Woessner, 1998).

1.1.5.2 Regulation of MMPs

The MMPs can be regulated at three levels: synthesis and secretion, activation of the pro-enzyme and inhibition of the active enzyme.

Synthesis and secretion of MMPs are regulated by cytokines and growth factors. IL-1 and TNFα stimulate synthesis and secretion of MMP-1, MMP-3, MMP-9 and MMP-13 and these MMPs are elevated in the synovial fluid of patients with arthritis (Mengshol et al., 2002). FGF and platelet derived growth factor (PDGF) can enhance secretion of IL-1-mediated MMP synthesis (Harvey et al., 1993). On the other hand TGFβ can inhibit the secretion (Chandrasekhar and Harvey, 1988). Other cytokines e.g. IL-4 and IL-10 can inhibit synthesis of MMPs (Lacraz et al., 1995).

Activation of proMMPs proceeds generally in a stepwise manner (Nagase, 1997) and it requires that the Cys-Zn^{2+} interaction is disrupted. The final activation step is usually catalysed by other MMPs.

Tissue inhibitors of metalloproteinases (TIMPs) are the major inhibitors of MMP activity in tissue (reviewed in Brew et al., 2000). They regulate the extracellular matrix degradation by inhibiting active MMPs and by inhibiting activation of proMMPs.

TIMPs are small (21-30 kDa) two-domain molecules, each domain being stabilised by disulphide bonds. Four TIMPs (TIMP-1 to TIMP-4) have so far been identified and they bind tightly to the active site of most MMPs, in a 1:1 stoichimetry. The TIMPs seem to lack specificity, but some differences in inhibitory effects have been described. For instance, TIMP-2 and TIMP-3 are effective inhibitors of MT-MMPs but TIMP-1 is not. TIMP-3, but not TIMP-1 or TIMP-2 has been shown to be a potent inhibitor of aggrecanase-1 and aggrecanase-2 (described in section 1.2) (Kashiwagi et al., 2001). TIMP-3 also differs from the other TIMPs by binding tightly to the matrix (Pavloff et al., 1992).

1.1.5.3 Involvement of interleukin-1 in cartilage degradation

Interleukin-1 is a primary inflammatory cytokine that plays an important role during inflammatory responses (Dinarello, 1991).

Much attention has been given to the role of IL-1 in chronic inflammatory diseases such as RA (Saklatvala et al., 1996). There are two types of IL-1, α and β, and although the two forms are distinct gene products, they recognise the same cell

surface receptors and share various biological activities. IL-1 is made by many cells (e.g. endothelial cells, B cells, and fibroblasts) but mainly by monocytes and macrophages in response to infection, microbial toxins, inflammatory agents, viruses and other cytokines like IL-2 and interferon γ. Local effects of IL-1 include leukocyte infiltration and accumulation at sites of injury or infection and the stimulation of production of prostaglandins that increase capillary permeability and enhance perception of pain. IL-1 upregulates expression of many genes, well-characterized examples being IL-6 and IL-8, cyclooxygenase-2 and the already mentioned MMPs. These changes are not specific to chondrocytes but are seen in other connective tissues cells such as fibroblasts.

IL-1 is present in arthritic joints, and the blood concentration correlates to the severity of RA (Badolato and Oppenheim, 1996). IL-1 has been found in inflammatory synovial fluid and is produced by cells in the synovial lining in RA patients and is involved in cartilage resorption. When injected into normal animal joints it causes degradation of proteoglycans and inhibits their resynthesis. Production of IL-1 inhibitors occurs simultaneously with IL-1 production (Weckmann and Alcocer-Varela, 1996) suggesting that antagonists are released together with IL-1. The IL-1 receptor antagonist protein (IRAP) has the same affinity for the IL-1 receptors, as IL-1 but does not induce a biological response. IRAP inhibits IL-1-induced cartilage damage in rabbit knees (Henderson et al., 1991).

IL-1 promotes degradation of cartilage by stimulating production of specific proteinases, which cleave collagen and proteoglycans. IL-1 inhibits the new synthesis of proteoglycans as measured by inhibition of sulphation of the glycosaminoglycan (GAG) side chains (Saklatvala et al., 96) and also inhibits type II collagen synthesis. Recently, it has been shown that IL-1 causes a rapid decrease in the levels of SOX9 in chondrocytes. SOX9 is a transcription factor required for cartilage formation and for expression of cartilage specific genes including the gene for collagen type II (Murakami et al., 2000).

Synthesis of the proforms of MMP1 and MMP 3 is induced by IL-1 but the physiological roles these enzymes play in cartilage destruction is still unclear.

In this project, stimulation of porcine chondrocytes with IL-1 was used as a working model to develop techniques to study secreted proteins by 1D- and 2D-electrophoresis and MS. Identification and characterisation of the protein components induced or upregulated by IL-1 in chondrocytes and the extracellular matrix could provide important information about how cellular activities are regulated during inflammation in RA.

Chondrocytes can be studied in isolated cell cultures or in explants where the cells are in their normal matrix. To what extent the phenotype of the cells and their responses to cytokines differ with respect to their being in a physiological matrix or in a monolayer culture is not known. Differentiation of chondrocytes occurs when cells are grown in monolayer with the production of collagen type I and III instead of collagen type II (Benya et al., 1977). Chondrocytes grown in monolayer culture differentiate into fibroblast-like cells after ca. 2 weeks (Shakibaei et al., 1997).

As the techniques were established they were also applied to studying the differences in protein expression between normal and OA cartilage.

1.2 Degradation of cartilage in disease

Loss of articular cartilage is a major feature of the two most common diseases of joints, RA and OA. Also in experimental models where animal cartilage is treated with IL-1, degradation of cartilage occurs (Saklatvala, 1987). One of the earliest changes observed is the depletion of proteoglycans (aggrecan). Proteolytic cleavage within the IGD of aggrecan is believed to be responsible for the loss of aggrecan from cartilage.

Many MMPs (MMP-1, -2, -3, -7, -8, -9 and –13 and MT1-MMP) cleave aggrecan between Asn^{341} and Phe^{342} within the IGD (Flannery et al., 1992; Fosang et al., 1998; Fosang et al., 1996; Fosang et al., 1993; Fosang et al., 1992). However it is the cleavage between Glu^{373} and Ala^{374} that has been mainly associated with disease. *C*-terminal fragments generated after cleavage at this site have been identified in synovial fluid from patients with knee injuries and OA (Lohmander et al., 1999), inflammatory joint disease (Lohmander et al., 1993) and in the medium from cartilage explants stimulated with IL-1 (Sandy et al., 1991). Two proteases have been identified and cloned that cleave aggrecan between Glu^{374} and Ala^{374}, aggrecanase-1 (ADAMTS-4) (Tortorella et al., 1999) and aggrecanase-2 (ADAMTS-11) (Abbaszade et al., 1999). These zinc proteinases belong to a subfamily of the disintegrin and metalloproteinase (ADAM) family of proteases that also contain a thrombospondin motif responsible for extracellular matrix binding.

Some controversy exists around the importance of aggrecanases. For example, Lark et al, 1997 found both MMP- and aggrecanase- generated fragments in normal, OA and RA articular cartilage. In the normal specimens, the fragment quantity increased with age.

In a more recent study, the concentration of aggrecanase-generated fragments was found to be lower in OA than in normal cartilage (Bayliss et al., 2001a), suggesting that the fragmentation was part of the normal matrix turnover.

Collagen type II denaturation occurs initially in the superficial and transitional zones in OA cartilage, extending to the deeper zones with disease progression (Hollander et al., 1995). Damage is always first seen around the chondrocytes,

implicating them in the degradation process. Collagen denaturation also occurs with ageing.

1.2.1 Rheumatoid arthritis

Rheumatoid arthritis affects about 1% of the population worldwide, predominantly women. There is chronic inflammation of the lining of synovial joints with infiltration of macrophages and lymphocytes; polymorphonuclear leukocytes are abundant in the synovial fluid. The synovium proliferates with formation of pannus, which spreads over the cartilage. Destruction of cartilage is believed to be mainly mediated by cytokine induction of destructive enzymes such as the MMPs (Feldmann et al., 1996).

1.2.2 Osteoarthritis

In OA (a much more common disorder than RA) there is degradation of cartilage without the severe inflammation seen in RA. Its cause is not understood and it is often thought to be a result of "wear and tear". It is a multifactorial disease and is more common with advancing age. Prevalence studies show that most people over the age of 65 have some OA. While in RA, cartilage is destroyed because of its proximity to inflammation, in OA the tissue is thought to fail as a result of a primary disturbance of chondrocyte function leading to failure to maintain a normal matrix. Whether there is primarily a failure of matrix synthesis or excess degradation or both is unknown.

OA is classified into primary (or idiopathic) disease when it occurs without any obvious previous insult to the joint. OA following an abnormality or injury is called secondary OA. In most cases however both primary and secondary factors are important. Risk factors can be divided into those that increase the susceptibility to OA and mechanical factors due to abnormal use of specific joints. Obesity, family history and age are clearly associated with development of OA. Smoking and hypermobility have also been suggested to increase the risk.

Trauma of the joint that leads to articular incongruity and recurrent instability is a common cause of OA. A relationship between occupational activity and certain types of OA is also well established. Thus, there is an increased risk of OA in the knee in people in knee-bending occupations such as miners, concrete workers and road workers, compared to office staff. The relationship between OA and sporting activities are less clear. There is a higher prelevance of knee OA in football players and hand OA in boxers. However it is not clear whether this is due to repetitive loading of the joint or to repetitive joint injury. The joints most commonly affected by OA are fingers, hip, knee and spine.

Disease progression is generally slow. Early OA is asymptomatic. No consistent OA markers are currently available to diagnose the disease prior to detectable cartilage degradation (with x-ray scan). As the disease progresses, clinical features of the OA joint become apparent with use-related pain, stiffness and loss of movement. These symptoms often lead to severe disability and handicap. Treatment is aimed at symptom control. Patients with severe OA generally have surgery to replace the degraded cartilage and subchondral bone with metal or plastic implants.

1.2.2.1 Pathology of osteoarthritis

The main pathological changes in the joint include damage to the articular cartilage, increased activity in the subchondral bone and formation of osteophytes at joint margins (fig 1.2). The osteophytes produce cartilage that contain a large amount of collagen type I but their role in OA and cartilage degradation is unclear (Mankin and Brandt, 1997). In advanced OA there are also synovitis formation, thickening and remodelling of subchondral bone, cyst formation and a marked narrowing of the joint space.

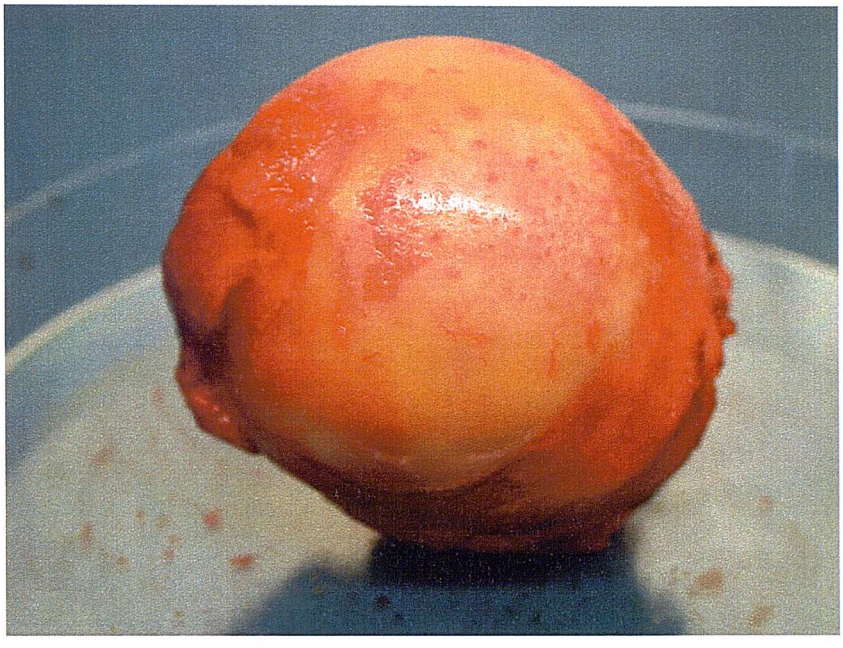
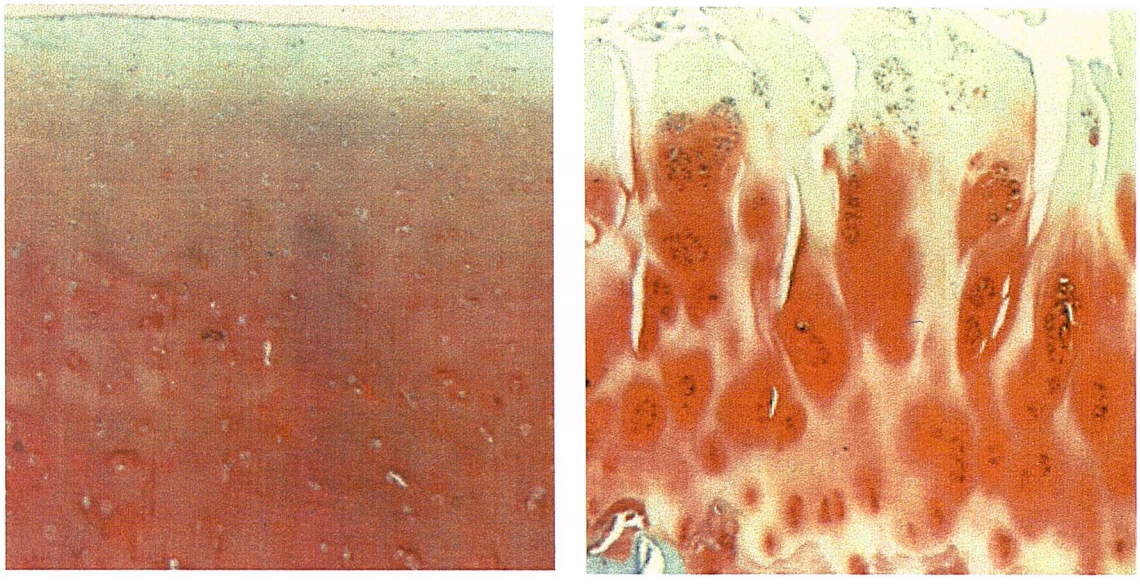


Fig 1.2. A typical OA femoral head obtained after replacement surgery of a hip joint from a 55 year old woman. Eroded areas exposing the underlying bone are clearly visible. The cartilage is yellowish.

1.2.2.2 Morphological changes in osteoarthritic cartilage

The articular cartilage surface is disrupted and fragmented with pitting, clefts and ulceration. In early OA this is confined to areas of the greatest pressure and movement. With advancing disease, more extensive degradation of the cartilage occur. The fibrillation initially confined to the superficial layer of cartilage, spreads more deeply and ultimately all cartilage has been degraded and the underlying bone is exposed (Collins, 1949).

Histological staining of articular cartilage for proteoglycans shows that in OA, the distribution of proteoglycans is uneven and the cells lie in clusters or possibly clones (fig 1.3) close to the sites of damage. The area surrounding the cell clusters is relatively rich in proteoglycans.



a b

Fig 1.3. Articular cartilage from a normal (a) and an osteoarthritic (b) knee. Proteoglycans of the matrix are stained with safranin O except for the surface where fine fibrils are stained with fast green. Image from Schubert and Hamerman, 1968.

1.2.2.3 Osteoarthritis and cell death

Cell death of chondrocytes can be caused by necrosis or apoptosis (programmed cell death). Necrosis is a pathological cell death caused by injury, exposure to toxins, severe hypoxia or hyperthermia. Since mononuclear phagocytes are not present in cartilage, dead cells will probably remain in the matrix. This could affect the matrix structure and the function of viable cells. As mentioned previously the cells are very sparsely distributed, and in areas with cell death, repopulation will occur slowly or not at all. This will impair the ability of cartilage to repair itself at damage.

Apoptosis may contribute to the development of OA. High frequencies of apoptotic cells were found in the cell clusters of OA cartilage (Hashimoto et al., 1998a). The locations of apoptotic cells have been shown to be related to areas of proteoglycan desorption. Also grading of histological changes in OA cartilage and analysis of apoptotic cells revealed a significant correlation between OA grade and

cell apoptosis (Hashimoto et al., 1998b). However, other groups have detected more modest amounts of apoptotic cells in OA cartilage and could not establish a correlation between apoptosis staining and cell morphology (Kouri et al., 2000; Aigner et al., 2001).

The apoptosis of chondrocytes in OA could be induced by Fas antigen, which upon binding to antibodies induces apoptosis. Fas antigen has been detected in the superficial and mid-zone of cartilage (Hashimoto et al., 1997).

Nitric oxide (NO) can induce apoptosis. Human articular chondrocytes stimulated with IL-1 or TNFα produce high levels of NO but it does not lead to apoptosis of the cells. In the presence of oxygen radical scavengers however, IL-1 was shown to induce apoptosis in chondrocytes (Blanco et al., 1995).

Loss of cell-matrix interaction and prevention of cell adhesion can initiate apoptosis (Meredith et al., 1993). Another way NO may contribute to apoptosis is by interference with the survival signals from the extracellular matrix. NO has been shown to interfere with cell migration and adhesion in cartilage (Frenkel et al., 1996).

A more recent paper suggests that NO is not cytotoxic and that NO-donor compounds may mediate apoptosis via toxic products other than NO (Del Carlo et al., 2002).

1.2.2.4 Biochemical changes in osteoarthritic cartilage

It has been suggested that the onset of OA is due to an increased influx of water due to defects in the collagen network. The reason for this is unknown but it enables the proteoglycans to swell to a greater degree of hydration than normal (Byers et al., 1977). The cartilage becomes softer and the collagen network fails to counterbalance the swelling pressure of the proteoglycans leading to further damage. Although the concentration of collagen is not altered in OA it has been shown that the fibers are shorter and less tightly assembled in OA (reviewed in (Mankin and Brandt, 1997)). As the disease progresses and cartilage is degraded, the water content decrease to eventually become lower than in normal.

Another early event is the decrease in proportion of keratan sulphate relative to chondroitin sulphate in proteoglycans (Mankin and Lippiello, 1971). The relevance of this finding is however unclear.

Initially there is an attempt at repair in cartilage with increased synthesis of proteoglycans and proliferation of cells. But as the disease progresses, the cartilage is depleted of proteoglycans and collagen type II. The increase in metabolic activity of the OA chondrocytes lead to new cartilage being laid down. This cartilage is a mixture of hyaline and fibrocartilage, containing type I and III collagen.

Very little is known about the mechanism and regulation of cartilage breakdown in OA. Studying protein expression in normal and OA cartilage could provide important information about chondrocyte behaviour and disturbances in disease. Using a proteomic strategy is appropriate since it is a hypothesis independent method of addressing the problem.

1.3 Introduction to Proteomics

Proteomics, defined as "the study of the total set of proteins encoded by a genome" (Wasinger et al., 1995), should provide fundamental insights into how cells function and are regulated. These could in turn help identify novel therapeutic targets.

Proteomics bridges the gap between genome sequence and cellular behaviour. It is impossible to predict from a gene sequence alone the exact nature and structure of an expected protein: there may be alternative splicing of the transcripts, and post translational modifications. Similarly, the association of proteins into complexes is not predictable. Proteomics is complementary to gene profiling methods in which mRNA levels are measured by means of gene chips. Gene profiling cannot predict amounts of protein produced. This depends on translational controls, post translational regulation and rates of degradation.

1.3.1 The use and development of 2D-electrophoresis

2D-electrophoresis was independently introduced by P.H O'Farrell and J. Klose in 1975. It uses different separation procedures in each dimension and can resolve several thousand proteins in the form of a two-dimensional map. In the first dimension the proteins are separated on the basis of their intrinsic charge, and in the second by their molecular size, resulting in a defined pattern of spots (fig 1.4). In the original technique the first dimension separation was performed in polyacrylamide gels cast in narrow tubes containing carrier ampholytes. The carrier ampholytes were mixed polymers that were not very well characterised and varied from batch to batch in manufacturing. The pH gradients were also unstable and had a tendency to drift to the cathode making the technique less useful for proteins of alkaline p*I*, and limited reproducibility of the first dimension separation. Because of the limitations of the carrier ampholyte separation, a technique using an immobilised pH gradient (IPG) was introduced (Bjellqvist et al., 1982). The immobilised pH gradient was created by covalently incorporating a gradient of well-characterised acidic and basic buffer groups into a polyacrylamide gel at the time it was cast. With the IPG gel strips, the

first dimension separation became more reproducible because the covalently fixed gradient did not drift and was stable over an extended pH range. The IPG gel strip also had a high loading capacity and was easy to handle since it was cast onto a plastic backing.

2D-electrophoresis provides higher resolution than 1D-electrophoresis although proteins that are poorly soluble (e.g. membrane proteins) may be better visualised on 1D-gels. Other limitations of 2D-electrophoresis are that very small, very large, very acidic or very basic proteins are difficult to resolve, and proteins may co-migrate in a single spot, in which case only the most abundant will be detected. Co-migration will also confound quantification by scanning densitometry. Reproducible protein patterns are difficult to achieve and this is dependent on careful sample preparation.

The resulting map of protein spots can be visualised with the Coomassie brilliant blue stain or the more sensitive silver stain. With silver staining the lower limit of detection of bovine serum albumin (BSA) is in the range 1-5 ng compared with around 50-100 ng with Coomassie brilliant blue. In silver staining the silver binds mainly to sulfhydryl and carboxyl groups of proteins.

The positional information of a protein on the gel cannot be translated directly into structure. Protein spots of interest can be excised from the gel, digested with trypsin and the resulting peptides analysed by mass spectrometry (section 1.4). With its high throughput and lack of requirement for prior knowledge of protein structure MS is a very useful method. Our experience is that 100 fmol of a protein on a gel is needed for identification, but the MS analysis is very sensitive to any form of contamination. Removal of silver prior to enzymatic digestion of silver stained protein samples enhances the sensitivity and quality of mass spectra (Gharahdaghi et al., 1999): presumably because the protein extraction is more efficient when the silver is removed. Proteins can be destained with Farmer's reducer, a mix of potassium ferricyanide and thiosulphate.

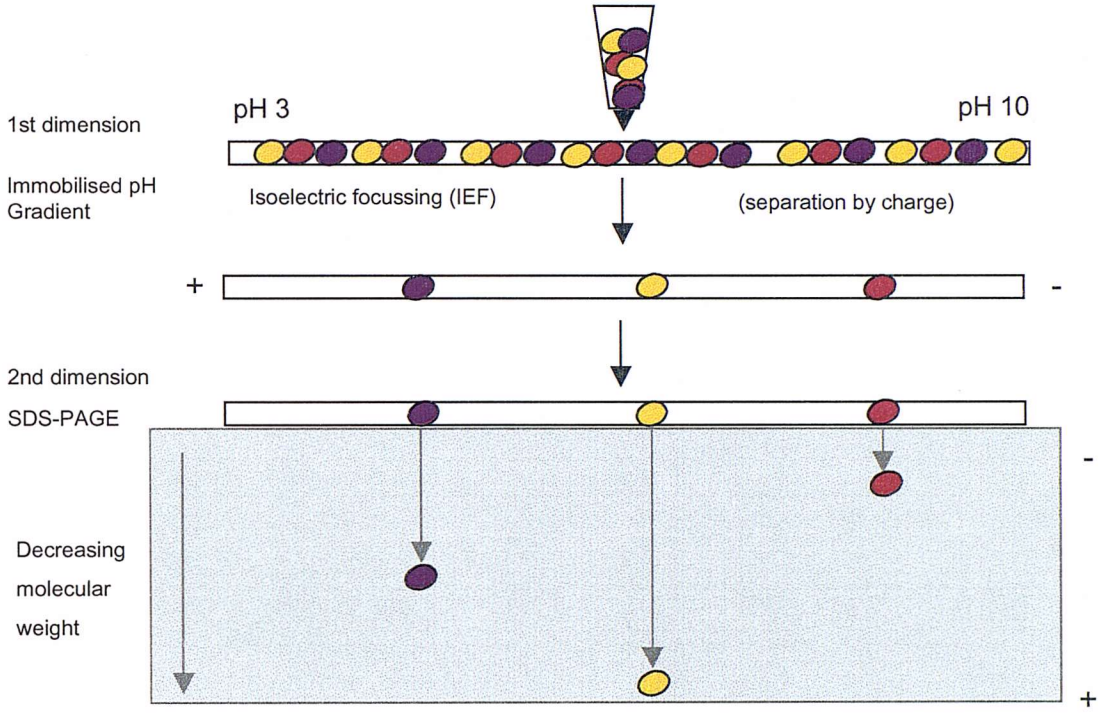


Fig 1.4. Principle of 2D-electrophoresis. Proteins (represented as different coloured ellipses) are applied to an immobilised pH gradient gel strip. In the first dimension they are separated according to their molecular charge by isoelectric focusing. The strip is put on top of a conventional SDS-PAGE gel and proteins separated in the second dimension according to their molecular mass.

1.4 Mass spectrometry

The mass spectrometer separates ionized molecules according to their mass-to-charge ratios (*m/z*) on the basis that ions of different mass and charge move differently in an electric field. If ionization deposits sufficient excess energy, a proportion of the sample ions will dissociate and form fragment ions. In the mass spectrum, the masses (strictly *m/z*) of intact molecular ions and fragmented ions are plotted against relative abundance (fig 1.5). Mass spectrometry gives a very accurate measurement of the molecular mass and has been used for exact determination of elemental compositions.

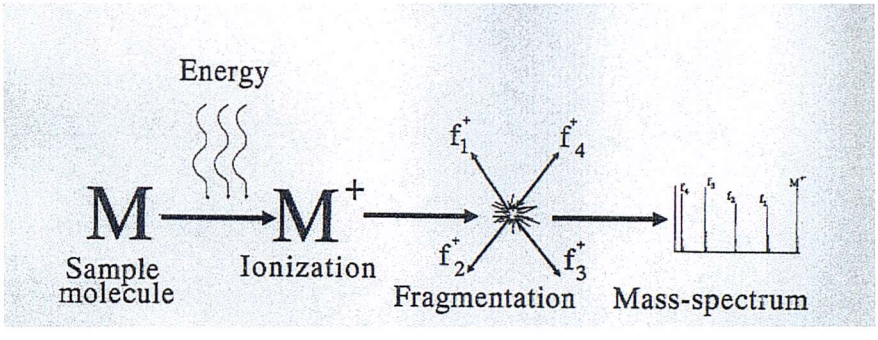


Fig 1.5. The principle of mass spectrometry. Sample molecules are ionized and a proportion of the molecular ions will be fragmented. The masses of the molecular ions and fragmented ions are determined and visualised as a mass spectra, where the masses are plotted against abundance. (Figure from Wait, 93).

1.4.1 A short history of mass spectrometry

Mass spectrometry was introduced early in the 20th century, and was initially used to determine the isotopic compositions of the elements. With the discovery that mass spectra could also be obtained from organic molecules, MS became an increasingly important technique in organic analysis in the years following the Second World War. However the requirement for thermal vaporization of samples limited its application to biopolymer analysis until the development of ionization methods which did not require heating of the sample, (initially plasma desorption (Macfarlane and

Torgerson, 1976), and fast atom bombardment (Barber et al., 1981)), subsequently and most importantly MALDI and ESI.

1.4.2 MALDI mass spectrometry

In the late 1980s two different solutions to the problem of vaporising and ionizing proteins were published: matrix-assisted laser desorption/ionization (MALDI) MS (Karas and Hillenkamp, 1988) and electrospray ionization (ESI) MS (Fenn et al., 1989). In MALDI ions are formed from sample molecules by laser-desorption from a highly UV-absorbing solid matrix (fig 1.6). The *m/z* of the resulting ions are determined by means of a time of flight (TOF) analyser. In TOF analysers, mass-to-charge values are determined by measuring the time it takes for ions to move through a field-free region. Given a constant accelerating voltage, the flight time for an ion is inversely related to its *m/z* value.

TOF analysis is particularity suited to MALDI MS, because each laser pulse results in a discrete package of ions and provides the start signal for the time of flight measurement. The advantages of MALDI include high sensitivity, tolerance to buffer and salt contamination, fast data acquisition and simple and robust instrumentation. Problems with inaccurate mass determination and relatively low mass resolution have (Mamyrin, 1994; Mann and Talbo, 1996) been overcome with the introduction of delayed extraction and reflectrons. Mass analysis with delayed extraction, an old concept that has recently been rediscovered improves resolution as well as the signal-to-noise ratios (Wiley and McLaren, 1955). The use of reflectrons (electrostatic mirrors) are also important for higher resolution. Matrix surfaces made by fast evaporation have further improved the accuracy of mass determination (Vorm et al., 1994). Highly accurate peptide masses (< 20 ppm) can be obtained with MALDI MS. It is a very useful technique to quickly establish whether a sample is free of major contaminants and contains a known protein.

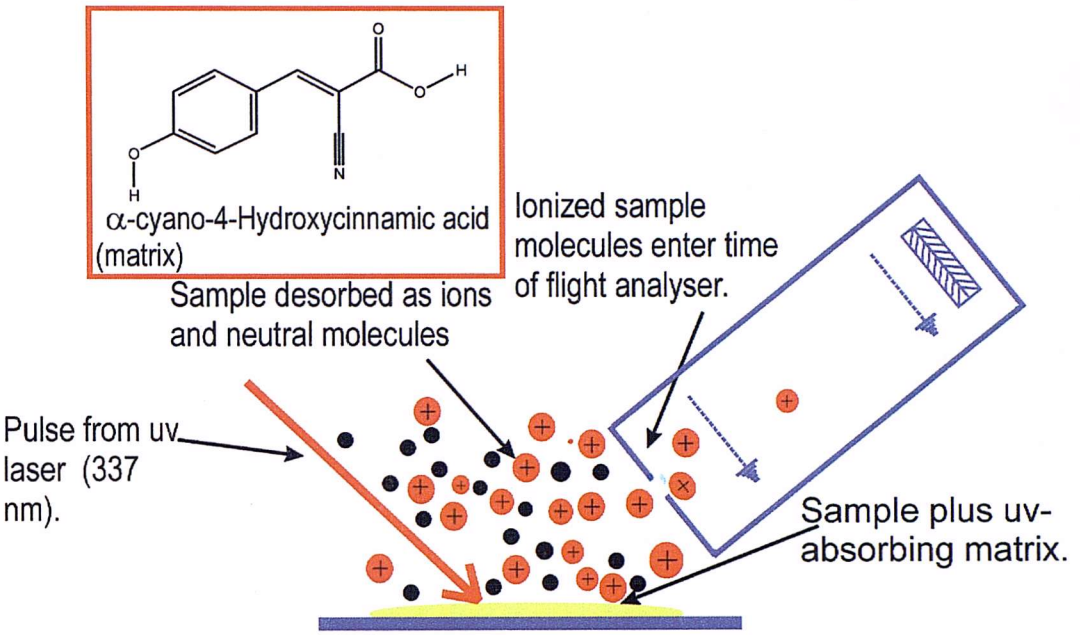


Fig 1.6. The principle of MALDI. A target plate is coated with a highly uv-absorbing matrix (HCCA) and the sample deposited. A pulse from an uv-laser (337 nm) is applied and ions are formed from the sample molecules. The ionized sample molecules are analysed by the TOF mass analyser.

1.4.3 Peptide mass fingerprinting

Peptide mass fingerprinting (Mann et al., 1993) using sequence databases allows unambiguous identification of proteins in subpicomole quantities. A protein sample is digested with trypsin and the resulting peptide masses are measured by MALDI MS. The masses of predicted tryptic peptides are calculated for each entry in the sequence database and compared with the experimentally measured peptide masses (fig 1.7). A correct identification will have a large number of peptide matches. High mass accuracy is important in obtaining large differences in the probability scores between the correct and false positive matches.

The MALDI/peptide mass fingerprinting approach is designed for matching to known proteins (or their very close relatives), and will not work if a protein is absent from a database. It is also likely to fail to identify low molecular weight proteins (<20 kDa), low abundance proteins and extensively post translationally modified proteins. Also mixtures containing 3 or more protein are difficult to identify with this approach.

If no protein is identified or if the result is ambiguous, the remainder of the peptide digest can be analysed by ESI tandem mass spectrometry (MS/MS) to obtain partial amino acid sequences, that can be used to search protein or expressed sequence tag (EST) databases (Mann, 1996; Wilkins et al., 1996).

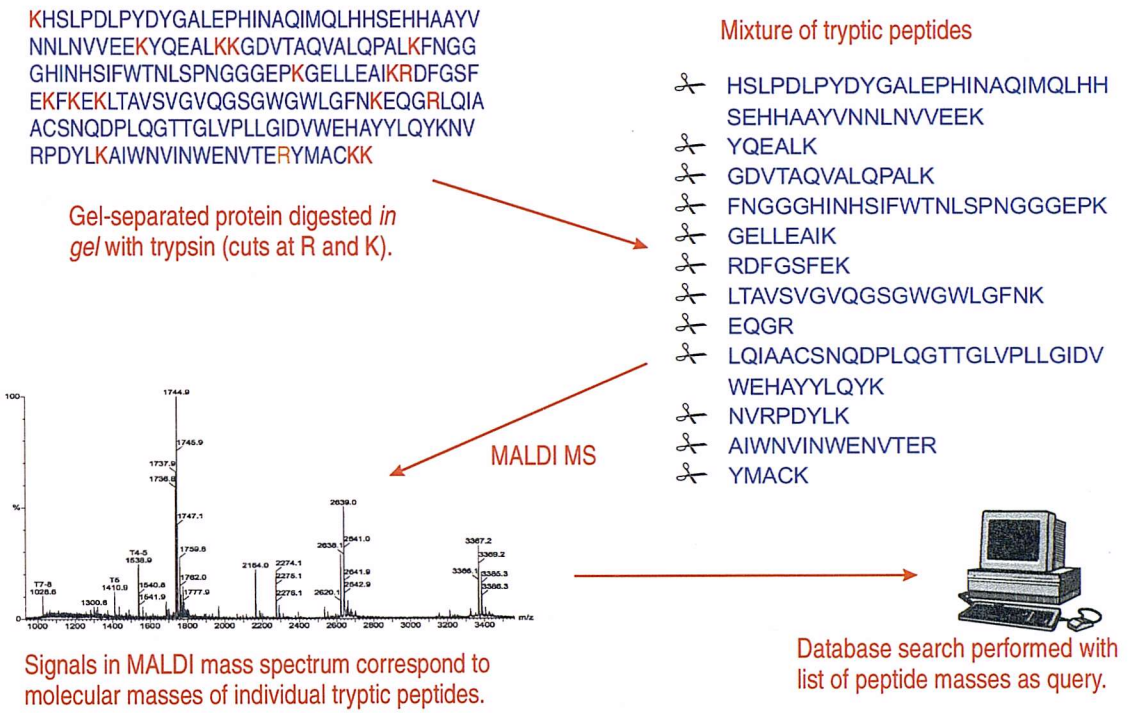


Fig 1.7. The process of peptide mass fingerprinting. Peptides generated from a protein sample by trypsin digestion are analysed by MALDI MS. The experimentally measured masses are compared with masses of theoretical digests of proteins in the database.

1.4.4 ESI mass spectrometry

The technique of ESI was actually first introduced by M. Dole in 1968 (Borman, 1998) but it was put into practice by Fenn (Fenn et al., 1989). In ESI MS, a solution of peptides or proteins is passed through a needle at high electrical potential. At the end of the needle the solution disperses into a mist of small, highly charged droplets. The small droplets shrink rapidly as the solvent evaporates and the ionized peptide or protein molecules are released into the gas phase (fig 1.8) and mass analysed. ESI typically produces multiple charged protein ions, in contrast to MALDI. This feature allows mass analysers with a limited mass-to-charge range to analyse quite large ions.

Shortly after the introduction of ESI, information was obtained from subpicomole amounts of protein. With the introduction of the nanoelectrospray ion source (Wilm and Mann, 1996), the lifetime of the sample was increased because the flow rate was very low (nl min^{-1}). The same amount of sample could be dissolved in 1µl solvent, while giving signal levels comparable to those in conventional ESI (flow rate $\approx$ 5µl min^{-1}). Reliable identification of proteins was thus extended to the femtomole level (Wilm et al., 1996), and ESI analysis of faint silver stained spots on 2D gels became possible for the first time.

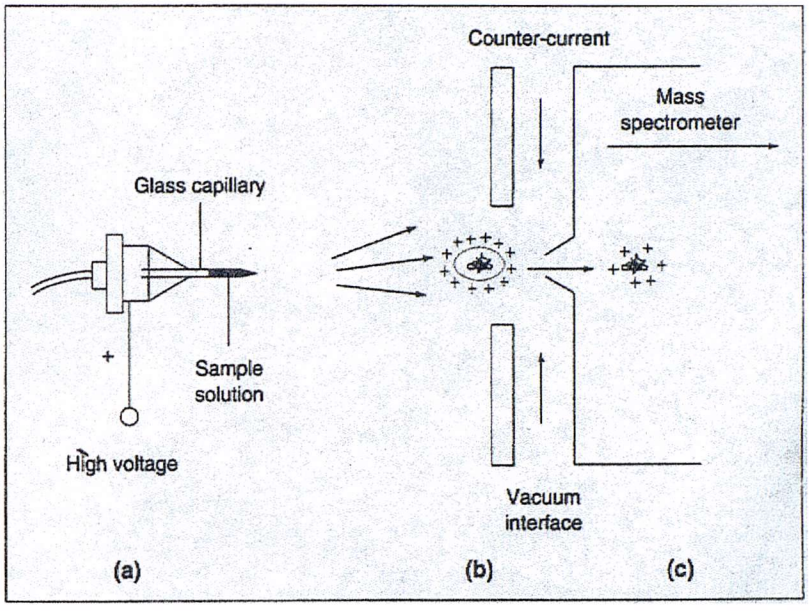


Fig 1.8. The three principal steps of electrospray ionisation mass spectrometry (ESI MS). A peptide solution is passed through a needle with a high electrical potential to create highly charged droplets containing the peptides (a). The droplets are evaporated rapidly by a counter-current of hot N_2 gas and the peptide ions are desorbed into the gas phase (b). The ions are mass analysed in a mass spectrometer (c). (Figure from Mann and Wilm, 95). .

1.4.5 Collisional activation and tandem mass spectrometry (MS/MS) of peptides

ESI is a gentle ionisation method that will not produce fragmentation of ionised molecules. To obtain structural information, ionised molecules can be fragmented by collision with a neutral gas such as argon (collision induced dissociation (CID)). This produces a spectrum characterised by a series of truncated peptide ions differing by the masses of successive amino acids, from which the sequence can be deduced (fig 1.9).

Different types of fragment ions are produced in the collision cell depending on the point of cleavage, and whether the charge is retained on the original amino or carboxyl terminal fragment. According to the Roepstorff and Fohlman system of nomenclature (Roepstorff and Fohlman, 1984), fragments in which charge is retained

on the *N*-terminus-derived product are named A, B or C depending on the cleavage point, whereas *C*-terminal fragments are similarly designated X, Y and Z (fig 1.10). The amino acid cleaved counting from the charge-bearing terminus is designated by subscripted Arabic numerals.

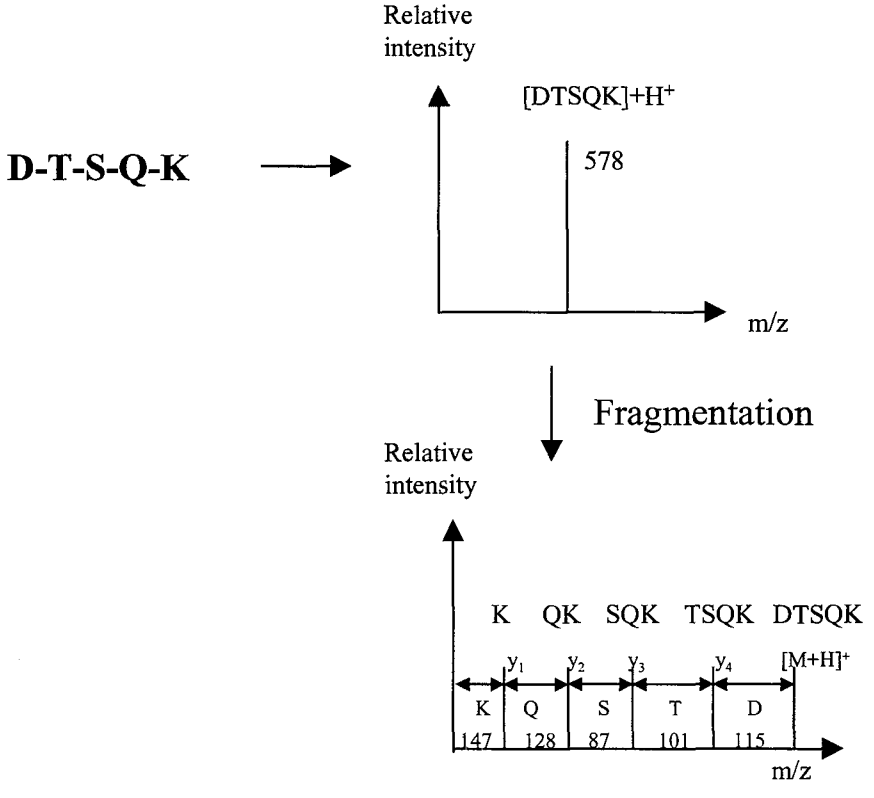


Fig 1.9. The principle of peptide fragmentation for identification of a peptide sequence. The protonated peptide D-T-S-Q-K is selected in the first analyser of a tandem mass spectrometer. The peptide is fragmented in a collision cell with argon into shorter fragments that differ in size by only one amino acid, which are separated in the second analyser. The masses of the fragment ions are determined and plotted against abundance. The fragment ions are all protonated. The mass difference allows identification of partial sequences that can be used to search databases. The masses of the different amino acids and the type of ion (y1-y4) are displayed.

The best way to record CID spectra is by tandem mass spectrometry, which employs an instrument that has two mass analysers in series. The MS/MS instrument used by us is a quadrupole-time-of-flight (Q-TOF) (fig 1.11), which has the advantage that it gives very high resolution and mass accuracy of the product ion spectra (the spectra obtained from fragmented ions). The quadrupole voltages are set so that only a single peptide mass is transmitted into the collision cell, where it is fragmented by collision with argon and product ion spectrum recorded with the orthogonal positioned TOF analyser.

1.4.6 Database searching with MS/MS data

The spectrum can be compared by computer to the fragmentation pattern predicted for known proteins from sequence databases. Since only one peptide spectrum is needed (compared to at least six peptide masses for MALDI), it is possible to match unknown proteins to highly divergent database homologues, containing only short regions of local identity. This also means it is easier to identify proteins in mixtures.

If direct searching of the peptide spectra against a protein database fails to retrieve any significant hits, amino acid sequences can be manually deduced from the mass spectrum (fig 1.9), and the deduced sequences can be used to query the database. The procedure can be repeated with several peptides from the same peptide digest. This will retrieve similar but not necessarily identical proteins. Recently, an algorithm has been described that can search several MS-derived peptide sequences simultaneously, evaluating all possible arrangements of the peptides (Mackey et al., 2002). This algorithm, called FASTS is designed to match derived amino acid sequences from organisms that lack complete proteome sequence data e.g. mammals other than human and mice.

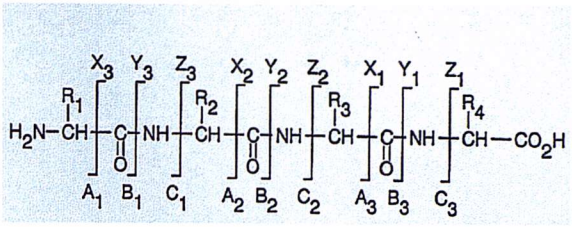


Fig 1.10. Different types of ions are obtained with fragmentation depending on where the sequence is cleaved. According to the Roepstorff and Fohlman system, fragments in which the charge is retained on the N-terminus product ("the beginning of the protein") are named A, B or C. If the charge is retained on the C-terminal product ("the end of the protein") the ions are designated X, Y or Z. The residue at which cleavage occur is indicated by a number and the fragments are numbered in ascending order from the charge retaining terminal.

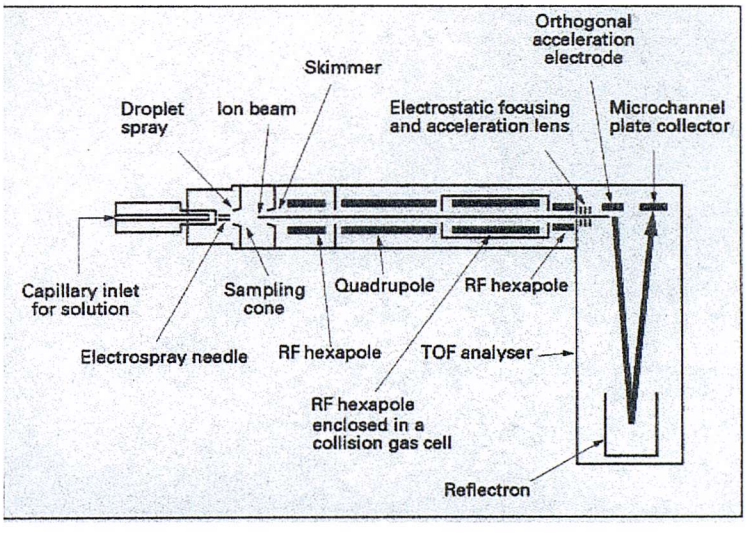


Fig 1.11. A schematic diagram of a Q-TOF instrument. An ion beam is produced by ESI. The RF hexapole is used to help confine the ions into a narrow beam. In the quadrupole, ions of a specific m/z value are selected. The ions fragment in the gas cell following collision with gas molecules. The TOF analyser is used to produce the final mass spectrum. Figure is from Micromass instruction manual for Hybrid Quadrupole-TOF.

If the protein is novel, enough sequence information can sometimes be obtained to allow cloning of the gene. MS/MS provides structural information by establishing relationships between the precursor ion and the fragmentation products.

Most eukaryotic proteins are post translationally modified. Product ion MS/MS spectra can provide information about modifications, however interpretation of the spectra was difficult and time-consuming. In 1995, Yates (Yates et al., 1995a) developed a computer algorithm to correlate tandem mass spectra of modified peptides to amino acid sequences in protein databases. A match can serve both to determine the sites of modification and identify the protein from which the peptide derived. The same group also developed a method to correlate tandem mass spectra of modified and unmodified peptides directly to sequences in nucleotide databases (Yates et al., 1995b).

The protein identifications presented in this work was obtained with Protein Probe and ProteinLynx Global server (Micromass). Second pass searches were made with Mascot software (Perkins et al., 1999). This search engine is freely accessible over the Internet. It uses a probability based scoring algorithm to match experimental data with proteins in the database. Thus the score will actually reflect a true probability. The advantage of Mascot is that a simple rule can be used to judge whether a match is significant or not. Also the score can directly be compared with those from other types of search, such as sequence homology. The software support searches of peptide molecular weights of a protein after enzyme digestion, MS/MS data from one or more peptides and a combination of mass data, amino acid sequence data and physiochemical data (size, p*I*).

Taken together, recent developments in 2D-electrophoresis and MS provide a powerful approach for separation and analysis of complex mixtures of proteins. These, together with expansion of sequence databases and improved search software tools have made it possible for proteins to be rapidly and accurately identified (fig 1.12). Much less time is needed for identification of a protein by MS than by Edman sequencing. In practice the method is at least an order of magnitude more sensitive for internal sequencing. The disadvantages of MS are that it is not possible to obtain

direct *N*-terminal sequence from intact proteins and that under the conditions used by us, MS will not distinguish between K/Q and L/I amino acids.

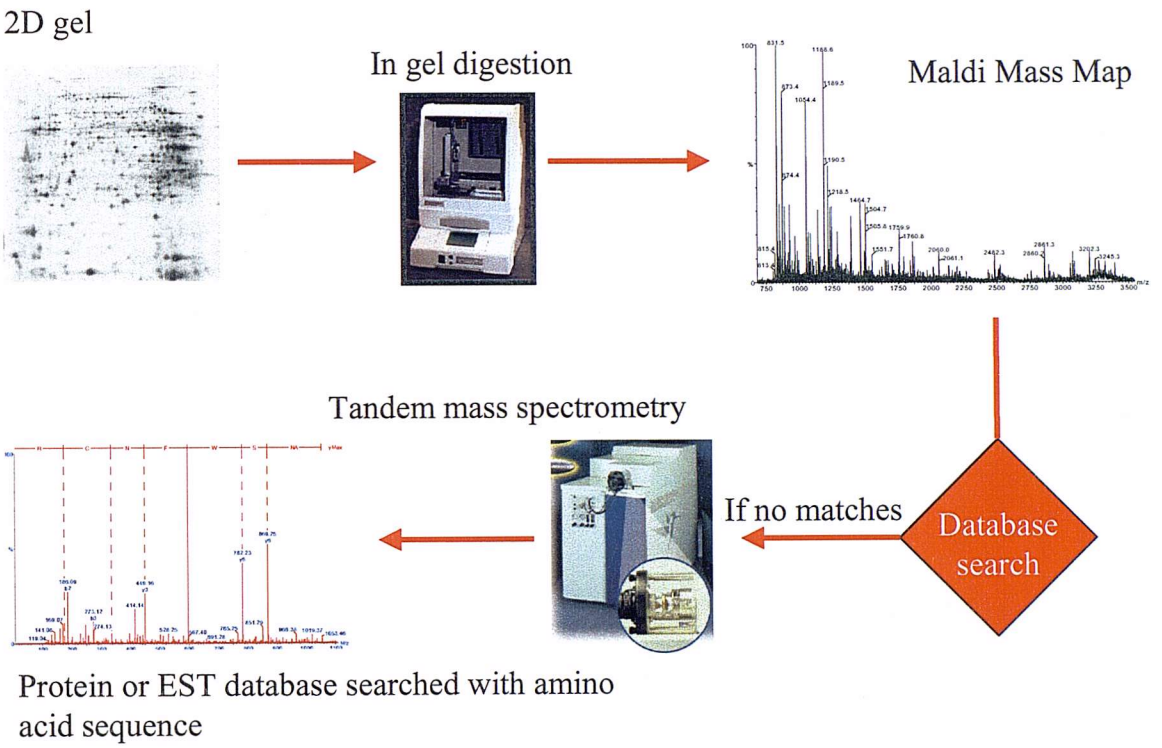


Fig 1.12. The process of protein identification using 2D-electrophoresis for protein separation and MS for protein analysis and identification.

1.4.7 Application of proteomics to biological studies.

Several examples of the application of proteomics can be found in the literature. Wasinger et al. '95 studied 2D gel-separated *Mycoplasma genitalium* proteins by MALDI-TOF and amino acid analysis. Identified proteins and amino acid sequences were linked to homologous genes in a variety of organisms. Clauser et al., 1995, separated proteins from human melanoma cells by 2D-electrophoresis and identified 11 proteins by MALDI and Edman degradation. Shevchenko et al., 1996 (Shevchenko et al., 1996a) successfully analysed 150 proteins in yeast and showed that proteomics could be used to link genome and proteome information for studies of the functions of uncharacterised open reading frames.

More recently, proteomics has been used to study differences in gene expression in bovine ventricle between normal animals and those with dilated cardiomyopathy. 35 proteins with altered abundance were found of which 12 were identified (Weekes et al., 1999). Correlation between protein and mRNA abundance in yeast was studied by Gygi et al. in 1999 (Gygi et al., 1999a) who showed it was insufficient to predict protein expression levels from quantitative mRNA data, especially from low abundance mRNAs.

Large scale identification of proteins of whole cells or tissues is technically feasible, however for the purpose of comparing protein expression between two cell states not necessary. Differences in protein expression would be of newly synthesised proteins, detectable by metabolic labelling. Due to the limitation of starting material though, many proteins will be present in very low amounts and beyond the reach even of MS analysis. Another problem is that very abundant proteins limit the amounts of material that can be loaded on a gel. This prevents analysis of proteins present in low abundance. A realistic task for proteomic analysis would be to identify all the proteins secreted by a cell: this would mean resolving and analysing perhaps one to two hundred protein spots. It seemed timely to apply these techniques to analysis of cartilage, a tissue consisting largely of extracellular matrix and having only one cell type.

Many academic research groups and pharmaceutical companies are currently using a proteomic approach to study protein expression in chondrocytes, synovial fluid and plasma to study joint diseases. However, very little has so far been published in peer-reviewed journals. Sinz et al, 2002 has compared protein expression in synovial fluid and plasma from patients with OA and RA using 2D-electrophoresis. This work and related publications are discussed in section 6.3.

1.5 Aim of project

To develop methods using 1 and 2D-electrophoresis and mass spectrometry to investigate the patterns of proteins secreted by cultured chondrocytes and explant cartilage. These methods will be applied to investigate differential expression of proteins in response to IL-1 stimulation, and to compare the effect of physiological surrounding (explant vs. monolayer) on protein synthesis.
Finally, healthy human and OA cartilage protein expression will be compared, with the aim of identifying potential disease markers.

Chapter 2

Materials and Methods

2 General reagents

All general laboratory reagents were from Sigma (Poole, Dorset, UK) or BDH (Poole, Dorset, UK) unless otherwise stated.

2.1 Cytokines and growth factors

The cytokines and growth factors used in this study are listed in Table 2.1.

Table 2.1 Recombinant cytokines and growth factors.

Cytokine/growth factor	Species	Source
Interleukin-1 α (IL-1)	Human	Prof. Jeremy Saklatvala
Interleukin-17 (IL-17)	Human	Schering-Plough, Dardilly, France
Tumour necrosis factor-α (TNFα)	Human	BASF Bioresearch Corporation, MA, USA
Basic FGF 154 amino acid (bFGF)	Human	Peprotec EC Ltd. London, UK
Transforming growth factor-β (TGFβ)	Human	Sigma, Poole, UK
Epidermal growth factor (EGF)	Human	R and D systems, MN, USA
Insulin-like growth factor 1 (IGF-1)	Human	Upstate Biotechnology, NY, USA
Platelet derived growth factor (PDGF)	Human	AM Pharmacia Biotech
Vascular endothelial growth factor (VEGF)	Human	R and D systems, MN, USA
Bone morphogenic factor-2 (BMP-2)	Human	Genetics Institute Inc., MA, USA
Bone morphogenic factor-4 (BMP-4)	Human	Genetics Institute Inc., MA, USA

2.2 Enzymes

Enzymes used in this study with their sources are listed in Table 2.2.

Table 2.2 Enzymes

Enzyme	Biological source	Source
Pronase E	*Streptomyces griseus*	BDH, lab supplies, Poole, UK
Collagenase	*Clostridium sp.*	Sigma, Poole, UK
Chondroitinase ABC lyase	*Proteus vulgaris*	Seikagaku, Kogyo, Japan
Chondroitinase ABC lyase	*Proteus vulgaris*	Sigma, Poole, UK
Keratanase 1	*Pseudomonas sp.*	Seikagaku, Kogyo, Japan
Trypsin	Porcine	Sigma, Poole, UK
Trypsin (modified)	Bovine	Promega, WI, USA
Hyaluronidase	Bovine testes	Sigma, Poole, UK

2.3 Inhibitors

The inhibitors used in this study and their source are listed in Table 2.3

Table 2.3 Inhibitors

Inhibitor	Specificity	Source
Aprotinin	Serine protease	Sigma, Poole, Dorset, UK
E-64	Cysteine protease	Sigma, Poole, Dorset, UK
Leupeptin	Serine/cysteine protease	Sigma, Poole, Dorset, UK
Pepstatin	Aspartic protease	Sigma, Poole, Dorset, UK
Phenyl methyl sulphonyl fluoride (PMSF)	Serine protease	Sigma, Poole, Dorset, UK
Sodium orthovanadate	Protein phosphatases	Sigma, Poole, Dorset, UK
IL-1 receptor antagonist protein (IRAP)	Antagonist for the IL-1 receptor	Prof. Charles Dinarello, USA
EDTA	Metal chelator	Sigma, Poole, Dorset, UK

2.4 Tissue and cell culture

2.4.1 Tissue culture reagents

Dulbecco's modified Eagle's medium (DMEM) was obtained as a 1x sterile solution from Biowhittaker (Verviers, Belgium) and was supplemented with 25 mM HEPES, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin (2 µg/ml). 1 % 100x ITS supplement media (containing insulin, transferrin and sodium selenite) (Sigma, Poole, UK) were added when explants were cultured for longer than 3 days.

Foetal calf serum (FCS) was purchased from Sigma (Poole, Dorset, UK) or Labtech. Aliquots of sera were stored at –20°C until required, then diluted into medium to give a final concentration of 10 %.

All tissue culture plastics were from Beckton Dickinson (Cowley, Oxford, UK), Nunc Life technologies Ltd. (Paisley, Scotland, UK) or Costar (NY, USA).

2.4.2 Cartilage explants

Articular cartilage was dissected from porcine metacarpophalangeal joints of 6 month old pigs within 24 hours of slaughter.

Human articular cartilage was obtained from surgical specimens following total hip, knee or shoulder replacements (for osteoarthritis, avascular necrosis or following trauma). Normal articular cartilage was also obtained from individuals undergoing below knee amputation for osteomyelitis or following trauma. Cartilage specimens were obtained with informed consent from patients undergoing surgery at Charing Cross Hospital, Hammersmith, London. Some normal cartilage specimens came from post mortem autopsies performed at Algemeen Ziekenhuis Sint-Jan, Bruges, Belgium or at the Department of Rheumatology, University of Ghent, Belgium. Due to the Belgian opt-out system, informed consent was not necessary for these specimens. All tissue samples used for analysis are listed in table 5.1.

2.4.3 Tissue culture

Cartilage explants (porcine: 5x10x1 mm, human: 5x5x3 mm approximately) were dissected into serum-free DMEM (1 ml/g cartilage) and supplemented as above. Explants were cultured for two days in serum-free medium with or without 1 % ITS supplement before use. The explants were stimulated with IL-1α (100 ng/ml) overnight (15-17 h).

2.4.4 Isolation of chondrocytes

Cartilage explants were left overnight following dissection. Chondrocytes were isolated from the cartilage by enzymatic digestion with 1 mg/ml/g cartilage of pronase E for 30 min at 37°C followed by 1 mg/ml/g cartilage of collagenase for 5 h at 37°C. The digest was strained then centrifuged at 1400 rpm for 8 min. Pellets were washed twice, then resuspended in DMEM containing 10 % FCS. Cells were counted and plated on 6 or 12-well plates (diameter of 35 mm or 22.6 mm) at a density of 2.5 or 5 million cells per well respectively (100 % confluent).

2.5 Monolayer chondrocytes

2.5.1 Cell culture

Isolated chondrocytes were incubated at 37°C / 5 % CO_2 in medium supplemented with 10 % FCS. After cells had been plated they were cultured overnight, or until confluent before use. The medium was changed every three days during culture. Chondrocytes were serum-depleted for 5 h by washing with serum-free medium prior to stimulation. This was done to remove growth factors present in the serum that could activate the cells. The cells were stimulated overnight (15-17 h) with IL-1 α (10-20 ng/ml) or with other factors.

2.5.2 Chondrocyte fractionation

Chondrocytes were scraped into phosphate buffered saline (PBS) and spun down at 500 g for 5 min. The PBS was removed and the cells suspended in 20 mM Tris buffer, pH 8. Cells were disrupted in a syringe with a 23 gauge needle and a 26 gauge needle

(three times with each). The sample was centrifuged at 16000 g for 10 min to pellet the membrane fraction. The supernatant, containing soluble cytosolic proteins was removed and the insoluble protein pellet was dissolved in urea lysis solution

2.6 Metabolic labelling of cultured chondrocytes and explants

PBS

4.3 mM Na_2HPO_4
1.4 mM KH_2CO_3
1.4 mM KCl
137 mM NaCl
pH 7.2

A 1x sterile solution of PBS was generated by diluting a 10x stock with sterile water.

Dissociative lysis buffer (RIPA)

1 % (v/v) NP-40
0.5 % (w/v) sodium deoxycholate
0.1 % (w/v) SDS
50 mM Tris, pH 7.4
150 mM NaCl
1 mM EDTA
10 mM sodium fluoride
25 mM β-glycerophosphate
10 mM tetrasodium pyrophosphate

Urea lysis solution

9.5 M urea
1 % (w/v) dithiothretiol
2 % (w/v) CHAPS
0.5-2 % carrier ampholyte buffer

The urea lysis solution was supplemented with freshly prepared 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml pepstatin and 10 µM E64 (or 10 mg/ml leupeptin) before use. Urea and carrier ampholyte buffer (0.5 % and 2 %) was purchased from Pharmacia Biotech.

Monolayer cells and explants were stimulated overnight. The next day cells were washed once with methionine and cysteine free DMEM for 30 min and then pulsed for approximately 5 h at 37 °C in methionine and cysteine free DMEM containing 25-50 µCi/well [^{35}S] methionine/cysteine. Explants were treated in the same way except that 200 µCi/ml/g cartilage was used.

After pulsing, the medium was removed and the cells were washed once with 1x PBS and then lysed in ice-cold RIPA buffer. Explants were extracted for 45 min at 4 °C in RIPA buffer. For 1D-electrophoresis conditioned media or extracts were mixed with 4x-SDS sample buffer and boiled for 5 min. Cells for 2D analysis were lysed in urea lysis solution. Cell medium was treated with hyaluronidase (see *2.7.2 Hyaluronidase treatment of cell medium*) or passed through an anion exchange column (see *2.7.3 Removal of polyanions from cell medium*), dialysed into water and lyophilized. Explant medium was prepared as cell medium but first depleted of proteoglycans, *(*see *2.7.5 Cetylpyridinium chloride (CPC) precipitation of explant medium)*. Protein concentrations were determined with the Bradford assay (*2.6.3*).

2.6.1 Anaerobic tissue culture

An anaerobic environment for explant culture was created with a modulator incubator chamber box 977 (Bilups-Rothenberg). AnaeroGen ^{TM} (Oxoid) containing ascorbic acid was used to generate anaerobic conditions in the incubator. DMEM supplemented with 25 mM HEPES, antibiotics and 1 % ITS was depleted of oxygen prior to culture. The explants were cultured for three days in the incubator prior to stimulation. Explants were stimulated with IL-1α (100 ng/ml) and left in the incubator over night. The following day the explants were washed with methionine and cysteine-free medium for 30 min in normal atmosphere and pulsed with [^{35}S]methionine/cysteine (200 µCi/ml) in methionine and cysteine-free medium for 5 h in the incubator. The explant medium was prepared for 2D-electrophoresis as described above.

2.6.2 Dialysis and lyophilization

All conditioned cell and explant medium samples were dialysed (10 kDa cut off) against water over night at 4°C or room temperature. Material was frozen and lyophilized using a Lyoprep-3000 freeze-drier (International Equipment Company, Dunstable, UK). The lyophilized residue was solubilised in urea lysis solution.

2.6.3 Protein determination by the Bradford assay (Bradford, 1976)

5 µl sample was mixed with 95 µl water and 900 µl Bradford reagent. The A_{595} of the sample was determined in a CE 292 digital spectrophotometer (Cecil Instruments, Cambridge, UK). Protein concentration was estimated by comparison of the A_{595} to a standard curve obtained using known concentrations of bovine serum albumin.

2.7 Cell and explant medium preparations for 2D-electrophoresis

Flow charts of the most frequently used procedures are shown in fig 2.1 (explant medium) and fig.2.2 (monolayer chondrocytes).

2.7.1 Assay for glycosaminoglycans

The proteoglycan content in a sample was estimated as chondroitin sulphate with the dimethyl methylene blue (DMB) assay (Farndale et al., 1982). 1,9-Dimethyl-Methylene blue came from Aldrich (Gillingham, Dorset, UK). The assay is based on the metachromatic shift in absorption maximum that occurs when the dye is complexed with sulfated GAGs. Whale and shark chondroitin sulfate (Sigma, Poole, Dorset, UK) was used as a standard and the results were expressed as µg GAG/ml conditioned medium. Measurements were done on a Labsystems Multiscan Biochromatic spectrofotometer (Thermo Life Sciences, Basingstoke, UK) at A_{540}.

2.7.2 Hyaluronidase treatment of cell medium

Cell medium was treated with 15 or 150 units of hyaluronidase/ml for 30 min at 37°C to digest hyaluronan.

2.7.3 Removal of polyanions from cell medium

Preswollen microgranular anion exchanger DE52 cellulose (Whatman) containing diethylaminoethyl (DEAE) charged groups was activated by washing the resin in 500 mM Tris, pH 7.5 containing 1.5 M NaCl for 3 h. Activated DE52 cellulose was stored in 50 mM Tris, pH 7.5, 150 mM NaCl and azide (0.02 %). The DE52 cellulose was packed into an Econo-Column chromatography column (10 cm x 1 cm) (Biorad, Hempstead, UK). The column was equilibrated with 3 column volumes of fresh DMEM of the same pH as the sample, and cell medium was loaded directly onto the column or after lowering the pH to 6.5 with diluted HCl. Material that did not bind to the column (run-through, RT) was collected and prepared for 2D-electrophoresis. Bound material was eluted with 0.5 M NaCl.

2.7.4 Chondroitinase ABC lyase treatment of cartilage explant medium

Explant conditioned medium were treated with chondroitinase ABC lyase (0.0025 u/10 µg GAG) for 1 h at 37 °C to remove chondroitin sulphate chains.

2.7.5 Cetylpyridinium chloride (CPC) precipitation of explant medium

Cetylpyridinium chloride solution (5 %) solution was prepared by dissolving CPC in water. An appropriate volume of 5 % CPC solution was added to the sample. After 30 min (unless otherwise stated) precipitation at room temperature, the samples were cooled for 1-2 h to precipitate excess CPC. The proteoglycan/CPC containing precipitate was spun down and removed. The pellet was washed once with cold 100 mM sodium chloride and the wash was pooled with the supernatant. Proteoglycans were recovered from the complex as follows. The pellet was washed with 0.4 M sodium acetate in 90 % (unless otherwise stated) ethanol to remove CPC, sodium acetate was removed with 90 % ethanol, and the remaining proteoglycan-rich pellet was mixed with 4x-sample buffer and boiled for 5 min prior to 1D SDS-PAGE.

2.8 SDS-PAGE

Samples were run on 10 or 12.5 % SDS-PAGE, silver-stained and dried (see *2.9.3 Gel drying*). Newly synthesised proteins were visualised by autoradiography and gels were rehydrated prior to excision of bands for mass spectrometric analysis.

4x-SDS sample buffer	0.4M Tris/HCl pH 6.8 40 % (v/v) glycerol 0.4 % (w/v) SDS 1 % (v/v) β-mercaptoethanol (omitted for non-reduced samples) 0.01 % (w/v) bromophenol blue
Stacking gel	125 mM Tris/HCl pH 6.8 5 % (w/v) acrylamide 0.13 % (w/v) bis-acrylamide 0.1 % (w/v) SDS 0.1 % (w/v) APS 0.1 % (v/v) TEMED
Separating gel	380 mM Tris/HCl pH 8.8 7 %, 10% or 12.5 % (w/v) acrylamide 0.19 %, 0.27 %, or 0.34 % (w/v) bis-acrylamide 0.1 % (w/v) SDS 0.07 % (w/v) APS 0.07 % (v/v) TEMED
Running buffer	25 mM Tris base 190 mM glycine 0.1 % (w/v) SDS

30 % Acrylamide / 0.07 % Bis-acrylamide was purchased from Scotlat (Severn Biotech Ltd, Worcs. UK). Only polished water with a resisitivity of >10 MΩ cm^{-1} was used for preparation and staining of gels.

2.8.1 1D-electrophoresis

Samples for 1D analysis were run on a 10 or 12.5 % SDS-PAGE gels (Laemmli, 1970) in the Cambridge electrophoresis apparatus (Cambridge Electrophoresis Ltd, Cambridge, UK). Samples were mixed with 4x-SDS sample buffer and boiled for 5 min before loading. Molecular weight markers were run along side the samples to allow sizing of proteins (Sigma Markers, Sigma, Poole, UK or SeeBlue markers, Novex, San Diego, CA, USA). Samples were run at either 45 mA/gel for approximately 3.5 h or at 8 mA/gel overnight. Gels were stained with either Coomassie brilliant blue or with a silver stain for higher sensitivity.

2.8.2 2D-electrophoresis

Equilibration buffer	150 mM Tris/HCL pH 8.8
	6 M urea
	2 % SDS (w/v)
	30 % (v/v) glycerol

Immobilised polyacrylamide gel (IPG) dry strips with a linear pH 3-10 gradient (Amersham Biosciences) were normally used for the first-dimension separation of 2D-electrophoresis. Strips with a linear 4-7 or 6-11 pH gradient were also used.

25-100 microgram of protein (determined by Bradford assay) was added to the individual lanes of an IPG strip rehydration tray (Amersham Biosciences) and the IPG strip positioned on top of the sample. Each IPG strip was overlaid with 2 ml silicon oil to minimise evaporation and urea crystallisation. The strips were allowed to rehydrate at room temperature overnight (>12 h).

Isoelectric focusing was normally performed using a commercial flatbed electrophoresis system, Multiphor II (Amersham Biosciences). The samples were run at 300 V for 1 min, then ramped to 3500 V for 1.5 h and then kept at 3500 V for 3.5 h.

For the IPG strips with a pH 6-11 gradient, the final phase of the program was increased to 23.5 h. After isoelectric focusing, IPG strips that were not used immediately were stored between two sheets of plastic film at -20 °C. Prior to second dimension separation the IPG strips were equilibrated for 15 min with 65 mM dithiothreitol (DTT) in equilibration buffer containing 2 % SDS, 6 M Urea, 30 % v/v glycerol and 150 mM Tris pH 8.8 to cleave disulphide bonds. Free SH-groups were alkylated by treatment with 260 mM iodoacetamide (IAA) in equilibration buffer for 15 min. Following equilibration the strips were transferred to a 10 or 12.5 % polyacrylamide second dimension gel (Laemmli, without stacking gel). Gels were run at 8 mA in a Protean® IIxi Cell apparatus (Biorad) until the dye front line was 5-10 mm from the bottom of the gel. Gels were removed and stained with either Coomassie brilliant blue or silver.

2.9 Gel staining

2.9.1 Coomassie brilliant blue

Coomassie brilliant blue stain	0.1 % (w/v) Coomassie brilliant blue G
	50 % (v/v) methanol
	20 % (v/v) acetic acid
Destaining solution	30 % (v/v) methanol
	1 % (v/v) formic acid

Gels were stained for 20 min, destained overnight, and dried prior to autoradiography. Gels were rehydrated and bands cut out for identification by mass spectrometry.

2.9.2 Silver staining

Gels were silver stained with a method compatible with mass spectrometric identification of proteins (Shevchenko et al., 1996).

The gel slab was fixed in 50 % methanol, 5 % acetic acid in water for a minimum of 20 min, followed by a wash with 50 % methanol in water for 10 min and with water alone for 10 min. The gel was incubated for 1 min with 0.02 % sodium thiosulphate

(sensitisation), rinsed with water twice, then incubated for 20 min in chilled 0.1% silver nitrate solution at 4 °C. The silver solution was discarded and the gel rinsed twice with water. The image was developed by adding 0.04 % formaldehyde in 2% sodium carbonate. Development was terminated by adding 5 % acetic acid (after 5-10 min). To aid rehydration, the gel was incubated overnight in 3 % glycerol, 30 % aqueous methanol prior to drying.

2.9.3 Gel drying

Gels were dried onto paper using a geldryer (model 583 Geldryer, Biorad).

2.9.4 Autoradiography

Dried radioactive gels were subjected to autoradiography using x-ray films (Hyperfilm ECL, Amersham Biosciences or Fuji medical x-ray film, Fuji Photo Film, Dűsseldorf, Germany). After 2-7 days, the films were developed on a Curix 60 film processor from AGFA-GEVAERT Ltd, Brentford, UK.

2.9.5 Quantification of protein expression

Autoradiographs were scanned on a GS-710 calibrated imaging densitometer (Biorad) Amounts of proteins on autoradiographs from human cartilage explant medium were estimated with Phoretix 2D software version 6.01 (Nonlinear Dynamic Ltd, UK). The area and intensity of each spot was measured as optical density (OD) and the value normalised against the value for the central TIMP-1 spot on the same autoradiograph (see e.g. fig 5.2). The amount of a specific protein on different autoradiographs were compared by first analysing the amount of the central TIMP-1 on all autoradiographs. All TIMP-1 values were then compared to one autoradiograph with a typical amount of TIMP-1 and the values multiplied or divided with a factor reflecting the difference from the this value, usually around 0.5-1.5. All other protein values were then corrected accordingly.

2.10 Chromatography of cell lysates

Ion exchange chromatography was carried out on Resource Q and S columns attached to an FPLC system (Amersham Biosciences) or on a Mono S column attached to a Amersham Biosciences SMART chromatography system. The SMART system was also used for gel filtration. All chromatography columns were from Amersham Biosciences (UK).

2.10.1 Filtration

All material was filtered using 0.2 µm sterile filters (Scleicher & Schuell, Dassel, Germany) before loading onto a chromatography column.

2.10.2 Chromatography buffers

Q buffer	20 mM Tris/HCl, pH 9.0 0.1 % (v/v) BRIJ 35
S buffer	sodium acetate, pH 5.0 0.1 % (v/v) BRIJ 35
Gel filtration buffer	1x PBS, pH 7.2 500 mM NaCl 0.1% (v/v) BRIJ 35

2.10.3 Resource Q anion exchange chromatography

A 6 ml Resource Q column was equilibrated with 5 column volumes of Q buffer. 12 ml of the insoluble fraction of IL-1 treated cell lysates were applied to the column. The material that ran through the column was collected (21 ml) and diluted five times with S buffer.

2.10.4 Resource S cation exchange chromatography

A 6 ml Resource S column was equilibrated with 5 column volumes of S buffer. The material that ran through the Resource Q column (102 ml when diluted) was loaded

onto the column and proteins were eluted with a 0-1 M NaCl gradient (15 ml). 1 ml fractions were collected. The eluted fractions were analysed by SDS-PAGE and proteins visualised by autoradiography.

2.10.5 Mono S cation exchange chromatography

A 0.1 ml Mono S column was equilibrated with 5 column volumes of S buffer. Fraction 7-9 from the Resource S column was pooled and loaded onto the Mono S column. Proteins were eluted from the column on a 0-1M NaCl gradient (1.7 ml). The 100 µl fractions were analysed by SDS-PAGE as described.

2.10.6 Size exclusion chromatography

A 2.4 ml Superdex 75 column was equilibrated overnight with gel filtration buffer. Fractions 4-6 from the Mono S column were concentrated (see *2.11.2 Enzyme digestion*) to 50 µl and injected onto the column. The material was pumped through the column at 40 µl/min. 15 fractions (100 µl) were collected and analysed by SDS-PAGE for detecting proteins of interest.

2.11 Neoepitope assay of aggrecanase

2.11.1 Aggrecan isolation

Aggrecan was purified by Ms Christie Gendron at the Institute. Bovine nasal septum cartilage was removed and frozen. 10 g of cartilage was sliced into small pieces and aggrecan extracted in 140 ml of extraction buffer (4 M guanidine hydrochloride in 50 mM sodium acetate, pH 5.8) for 40 h at room temperature. Aggrecan was further purified by equilibrium density gradient centrifugation in caesium chloride (1.5 g/ml extract) at 34,000 rpm for 48 h at 4°C. The bottom two-fifths of the centrifuge tubes contained aggrecan monomers. These fractions were collected and dialysed extensively against water at 4°C, lyophilized and stored at -20°C.

2.11.2 Enzyme digestion

Aggrecanase assay buffer	1x	4x
	50 mM	200 mM Tris pH 7.5
	150 mM	600 mM NaCl
	10 mM	40 mM $CaCl_2$

Confluent chondrocytes were stimulated with or without IL-1 (10 ng/ml) for 40-45 h in 6-well plates. The cells were cultured in 2.5 ml media per well. The medium was removed and 500 µl of each sample was concentrated down to 50 µl using a concentrator tube (YM-10 Microcon (10 kDa cut-off) from Amicon, Bedford, USA or Vivaspin 500, (5 kDa cut-off) from Sartorius, Epson, UK).

For each assay was used:	50 µl 4x aggrecanase buffer
	50 µl purified aggrecan (2 mg/ml)
	50 µl water
	50 µl sample (cell medium)

Each assay was performed in duplicates with 20 µl 500 mM EDTA added to one of the samples to control for non-metalloproteinase activity. The mixture was incubated

overnight at 37°C to allow aggrecanase in the samples to cleave the bovine aggrecan substrate. The next day, 10 µl 500 mM EDTA was added to stop the reaction.

2.11.3 Deglycosylation of aggrecan and aggrecan fragments

Prior to analysis of aggrecan fragments by Western blot, samples were first digested with chondroitinase ABC lyase (0.01 u/10 µg GAG) and keratanase (0.01 u/10 µg GAG) in buffer containing 50 mM sodium acetate, 100 mM Tris-HCl, pH 6.8 for 3 hr at 37°C. After digestion, the aggrecan was precipitated with 5 vol. of ice-cold acetone and mixed with 30 µl reduced 4x- SDS-sample buffer and boiled for 5min.

2.11.4 Neoepitope antibodies

Monoclonal antibody BC-3 was a gift from B. Caterson (University of Cardiff, Cardiff, UK). This antibody specifically recognises the new N-terminus, ARGSVIL, generated by cleavage of aggrecan between the Glu^{373} and Ala^{374} site. The size of this fragment is 250 kDa. Polyclonal GELE-antibody was kindly supplied by Dupont, Delaware, USA. This antibody is raised against the new C-terminus, ATTAGELE of the above fragment after cleavage between Glu^{1480} and Gly^{1481}. This antibody recognise the 250 kDa fragment as well as a 320 kDA fragment if aggrecan has not previously been cleaved between the Glu^{373} and Ala^{374} site. See image in fig 3.16.

2.11.5 Western blot with neoepitope antibody

Transfer buffer	25 mM Tris base
	190 mM glycine
	0.075 % (w/v) SDS
	20 % (v/v) methanol
10x Tris-buffered saline (10x TBS)	100 mM Tris pH 7.4
	1.5 M NaCl
	10 % azide

For use a 1x solution of Tris buffered saline/ Tween (TBS-T) was generated by diluting the 10x TBS with water and adding 0.05 % Tween 20.

Blocking solution	TBS-T 5 % (w/v) Marvel dried milk

Protein samples were loaded onto a 7 % polyacrylamide mini-gel and separated by SDS-PAGE electrophoresis at 125 V for 1.5 h in a Novex X cell Sure Look Electrophoresis Cell. Proteins were transferred onto a pre-hydrated polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using a Mini Transfer-Blot Transfer Cell (Biorad, Hemel, UK). Transfer was carried out at 30 V for 16 h with chilled transfer buffer. After transfer the membrane was washed for 30 min with TBS-T, blocked for 1 h with 5 % dried milk in TBS-T and incubated for 1 h with the BC-3 antibody diluted 1/1000. The blot was washed 3x 10 min in TBS-T, then incubated for 1 h with goat anti-mouse IgG alkaline phosphatase conjugated antibody diluted 1/5000. Blot was washed with water for 1 min and signal developed with Western Blue stabilised substrate for alkaline phosphatase (Promega, Madison, USA).

2.12 Western blot of aggrecanase

The same buffers were used as for western blot with neo-epitope antibodies but large gels were used and the antigen visualised by chemiluminescence.

2.12.1 Preparation of antiserum for aggrecanase

Three antisera against aggrecanase-1 (ADAM-TS4) (SAK29, SAK30, SAK31) were raised in sheep against three different peptides from human aggrecanase (Babraham Institute, Cambridge, UK). The sequences are listed in table 2.4. The SAK29 and 30 peptides correspond to sequences from the catalytic domain and SAK31 against the C-terminus of aggrecanase.

Table 2.4 List of antibodies raised against aggrecanase-1.

Antibody	Human sequence	Domain	Source
SAK29	CISLNGPLSTSRHV	Catalytic	Sheep
SAK30	PVMAHVDPEEP	Catalytic	Sheep
SAK31	LEILRRRPWAGRK	C-terminus	Sheep

2.12.2 Western blot

Protein samples were loaded onto a 12.5 % polyacrylamide gel and separated by SDS-PAGE as described (*see 1D-electrophoresis*). Proteins were transferred onto a PVDF membrane using a Trans-blot chamber (Biorad, Hemel, UK). Transfer was performed at 100 V for 1 hr or at 250 mA overnight. The transfer buffer was kept at 4°C by a water-cooling circulating pump. After transfer, the membrane was stained with Coomassie brilliant blue and destained with 60 % methanol. The blot was equilibrated in TBS-T for 10 min and then blocked for 1 h with 5 % dried milk in TBS-T. The blot was incubated for 2 h or overnight with the primary antibody diluted 1/1000. The blot was washed 5x 5 min in TBS-T, then incubated for 1 h with secondary horseradish peroxidase-conjugated antibody (1:2000). The membrane was washed 5x 5 min in TBS-T and signal developed by enhanced chemiluminescence (ECL, Amersham Biosciences, UK). Protein bands were visualised by autoradiography.

2.13 Mass Spectrometry

2.13.1 In gel digestion of proteins

In-gel digestion of silver-stained bands with trypsin was performed according to published methods (Jeno et al., 1995), (Wilm et al., 1996), (Shevchenko et al., 1996) modified for use with a robotic digestion system (Wait et al., 2001) (Investigator ProGest, Genomic Solutions, Huntington, UK). Excised gel pieces were washed with 50 mM ammonium hydrogen carbonate buffer and dehydrated with acetonitrile. Cysteine residues were reduced with DTT, and free SH-groups were alkylated with IAA. After further washing with ammonium hydrogen carbonate buffer, the gel pieces were again dehydrated with acetonitrile and dried at 60°C. This was followed by addition of modified trypsin (10 µl at 6.5 ng/ml in 25 mM ammonium hydrogen carbonate). Digestion was performed for 8 h at 37°C and peptides were recovered by sequential extraction with 25 mM ammonium carbonate, 5 % formic acid and acetonitrile. The pooled extracts were lyophilized and redissolved in 0.1 % formic acid prior to mass spectrometry.

2.13.2 MALDI mass spectrometry analysis

Spectra were recorded from α-cyano-4-hydroxycinnamic acid (HCCA) matrix (Beavis et al., 1992) prepared by fast evaporation according to Vorm et al. '1994 improved by Jensen et al. '1996 and modified by us.

Briefly, a saturated HCCA solution was prepared in acetone and mixed in a ratio 3:2 (v/v) with a 1 g/100 ml solution of nitro-cellulose blotting membrane in acetone/2-propanol (1:1,v/v). 0.12 µl of this solution was deposited in sample wells on a clean 100 well MALDI target plate. After fast evaporation of the solvent, 0.75 µl digest extract was deposited on the matrix film. Samples were allowed to dry and then desalted by washing briefly with 5 µl of cold 0.1 % trifluoroacetic acid before analysis.

MALDI mass spectra were obtained with a Micromass Tof Spec 2E time of flight spectrometer, fitted with delayed extraction source and a 337 nm nitrogen laser (Micromass, Manchester, UK). The instrument was operated in reflectron mode at 20 kV accelerating voltage, under the control of the MassLynx datasystem (v. 3.4). Spectra were either internally calibrated with trypsin autolysis product, or externally calibrated using Angiotensin I and $ACTH_{clip18\text{-}39}$ (Sigma A0673) as reference masses.

The resulting peptide masses were compared against a theoretical digestion of the NCBI non-redundant database, using Protein Probe (Micromass) or Mascot (Matrix Science) software. Internally calibrated spectra were searched to a tolerance of 50 ppm, which was increased to 200 ppm for externally calibrated data.

2.13.3 ESI mass spectrometry analysis

In early experiments, samples for ESI MS were desalted on C18 ZipTips (Millipore) or on home made microcolumns of Poros R2 (PerSeptive biosystems) Peptides were eluted in 0.5-2 µl of 50 % methanol/0.1 % formic acid and loaded into palladium coated borosilicate capillaries (Protana, Odense, Denmark) and installed in the nanoelectrospray source of a Micromass Q-Tof hybrid quadrupole orthogonal acceleration Tof spectrometer (Micromass, Manchester, UK). The capillary voltage was 900-1300 volts, and nitrogen was used as desolvation gas.

More recent spectra were recorded on the Q-Tof spectrometer interfaced to a Micromass CapLC capillary chromatograph. Samples were dissolved in 0.1 % aqueous formic acid, and 6 µl were injected onto a Pepmap C18 column (300 µm x 0.5 cm; LC Packings, Amsterdam, NL). The column was washed for 3 min with 0.1 % aqueous formic acid (with the stream select valve diverting the column effluent to waste). The flow rate was then reduced to 1 µl per min, the stream select valve was switched to the data acquisition position, and an acetonitrile - 0.1 % formic acid gradient (5 %-70 % acetonitrile over 20 min) was initiated to elute peptides into the mass spectrometer. The capillary voltage was set to 3500 V, and data dependent MS/MS acquisitions were performed on precursors with charge states of 2, 3 or 4 over a survey mass range 540-1000.

Trypsin autolysis products and major keratin derived precursor ions (contamination by the handler) were automatically excluded from MS/MS acquisitions. Precursor ions were fragmented by collision with argon and the collision voltage was varied between 18-45 V depending on the charge and mass of the precursor. To facilitate interpretation, the multiple charged daughter ion spectra were charge-state de-encrypted onto a singly charged mass axis, using a maximum entropy algorithm (MaxEnt 3, Micromass). Proteins were identified by correlation of uninterpreted tandem mass spectra to entries in SwissProt/TREMBL using ProteinLynx Global Sever (Version 1, Micromass). One missed cleavage per peptide was allowed, and an initial mass tolerance of 50 ppm was used in all searches. Cysteines were assumed to be carbamidomethylated, but other modifications were not considered in the first pass search. When this approach failed, amino acid sequences were deduced manually from the charge state de-encrypted spectra, and searched against the NCBI's non-redundant database using BLAST (Altschul et al., 1997) or FASTS (Mackey et al., 2002)

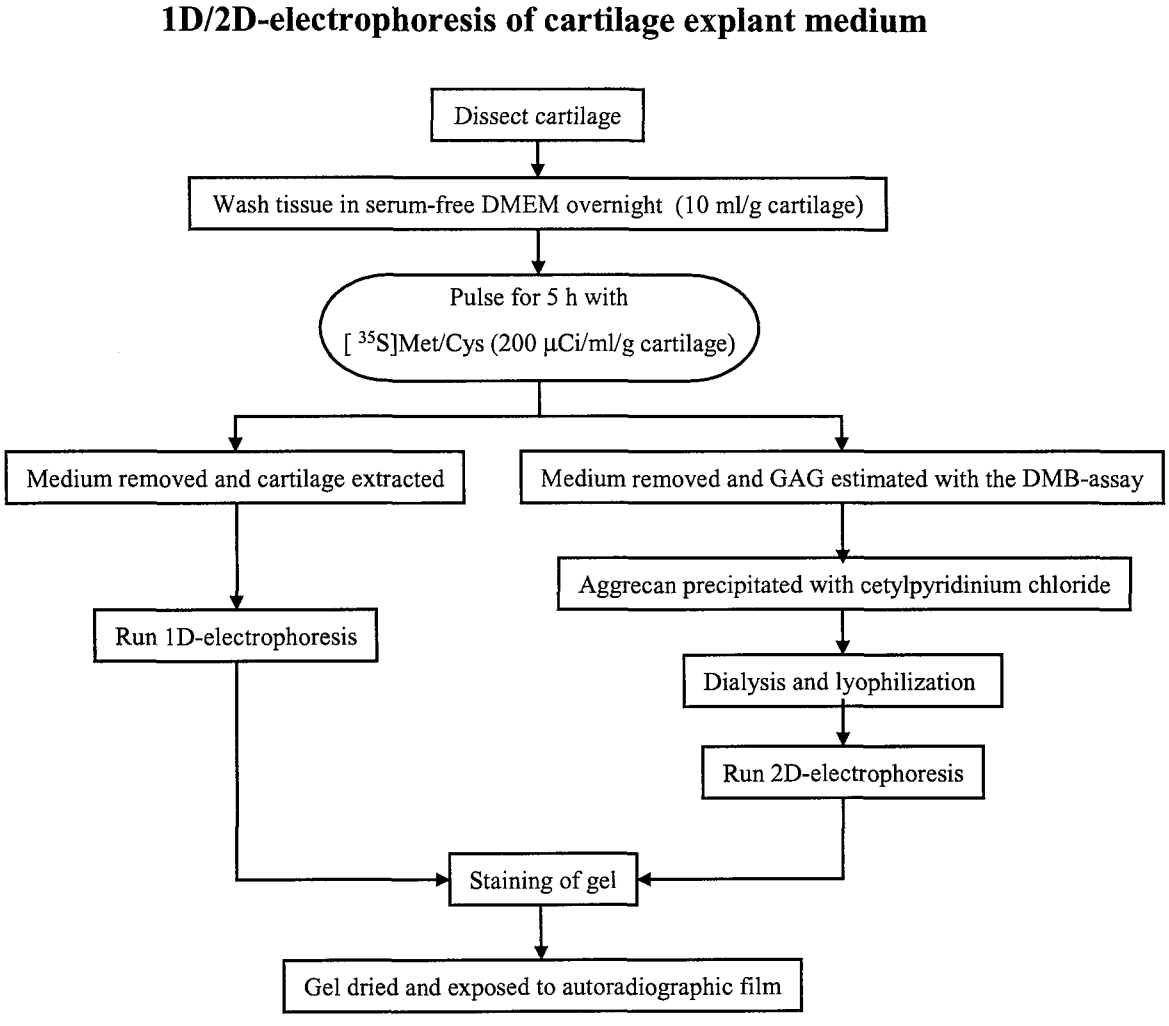


Fig 2.1 Flow chart of the most frequently used procedures for cartilage explant medium

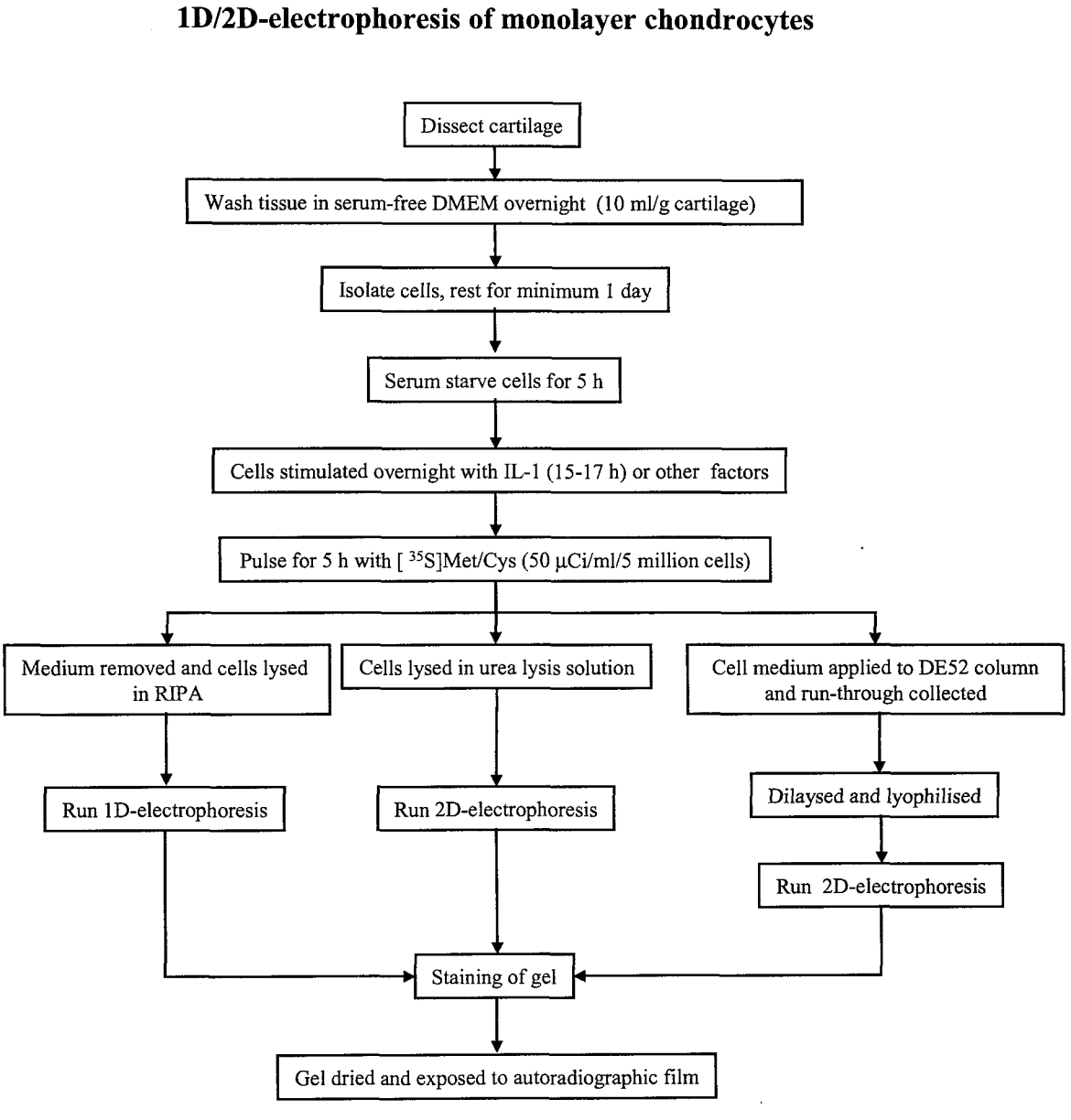


Fig 2.2 Flow chart of the most frequently used procedures for monolayer chondrocytes.

Chapter 3

Use of electrophoresis and mass spectrometry to identify chondrocyte proteins and to study their regulation

3 Introduction

This chapter describes early experiments in which proteins were identified by mass spectrometry after 1D- and 2D-electrophoresis. The potential of the technique was explored using IL-1 as a chondrocyte stimulus. Problems were encountered when trying to use 2D-electrophoresis to analyse the secreted proteins in the culture medium of both explants of cartilage and chondrocytes in monolayer. These problems and their solutions are described in chapter 4.

3.1 First experiments: effects of cell environment and IL-1 on chondrocyte protein expression

Initial 1D-electrophoresis experiments were carried out to study how the phenotype of porcine chondrocytes and their response to a strong activator (IL-1) differs when in their physiological matrix (cartilage explants) or compared to monolayer culture. Chondrocytes in monolayer culture should be easier to work with because of the relative lack of matrix components, particularly proteoglycans. Contamination with proteins from synovial fluid and other tissues like bone or blood would also be avoided. However, in pathological studies it is important to analyse chondrocytes in their physiological surrounding.

Porcine cartilage explants and serum starved chondrocytes in monolayer were either treated or untreated with IL-1 overnight (15-17 h) and pulsed with [^{35}S]methionine/cysteine for 5 h the following day. Conditioned medium samples, and lysates of cells and explants, were analysed by SDS-PAGE (fig 3.1). Gels were stained with Coomassie brilliant blue, dried and autoradiographed. The biosynthetic patterns of secreted proteins of the unstimulated cells and explants were distinctly different (fig 3.1a versus 3.1c: autoradiograph control lanes). The patterns of the lysates of cells and explants were as expected, very complex (fig 3.1b versus 3.1d: autoradiograph control lanes).

Five protein bands were strongly upregulated by IL-1 in both monolayer chondrocytes (fig 3.1a and b) and cartilage explants (fig 3.1c and d). The proteins

were approximately 14 kDa, 16 kDa, 23 kDa, 30 kDa and 40 kDa. The 14 kDa and 40 kDa bands were secreted proteins, whereas the others were cell associated. However, the 40 kDa band was found in the IL-1 treated explant extract as well as in the explant medium. The 23 kDa and 40 kDa upregulated protein bands were visible with Coomassie blue stain (fig 3.1a, b and c: stained IL-1 lanes), indicating that they were present in high levels and would be possible to identify by mass spectrometry. In chondrocyte medium (fig 3.1a) upregulated protein bands around approximately 55 kDa were also detected with autoradiography. Their sizes were consistent with their being MMPs. They were not detectable in explant medium.

The conclusion from these experiments was that there were differences in biosynthetic patterns of secreted proteins between explants and monolayer cells. However, the five proteins most strongly induced by IL-1 were very similar in both cases.

3.2 2D-electrophoresis of chondrocyte lysates

2D-electrophoresis was used to separate proteins upregulated by IL-1 in chondrocytes for subsequent identification by mass spectrometry. It was hoped that this technique would reveal more regulated proteins then were detected by 1D-electrophoresis.

Proteins in lysates of porcine chondrocytes cultured with or without IL-1 (10 ng/ml) overnight were metabolically labelled for 5 h, isoelectrically focused on an immobilised 3-10 pH gradient and separated in the second dimension by SDS-PAGE (10 % acrylamide). Approximately 200 resolved protein spots were visible on the autoradiographs (fig 3.2). The fine detail of the pattern of spots of both gels was very similar. The 23 kDa protein upregulated by IL-1 on 1D-gels (fig 3.1b) was also visible on the 2D-gel. However, no candidate spots were found for either the 16 kDa or 30 kDa proteins (of fig 3.1b). Possible reasons were that these were not soluble in urea buffer or that they failed to focus because of poor solubility or extreme p*I*, or that the transfer from the IEF strip to the 2^{nd} dimension was inefficient.

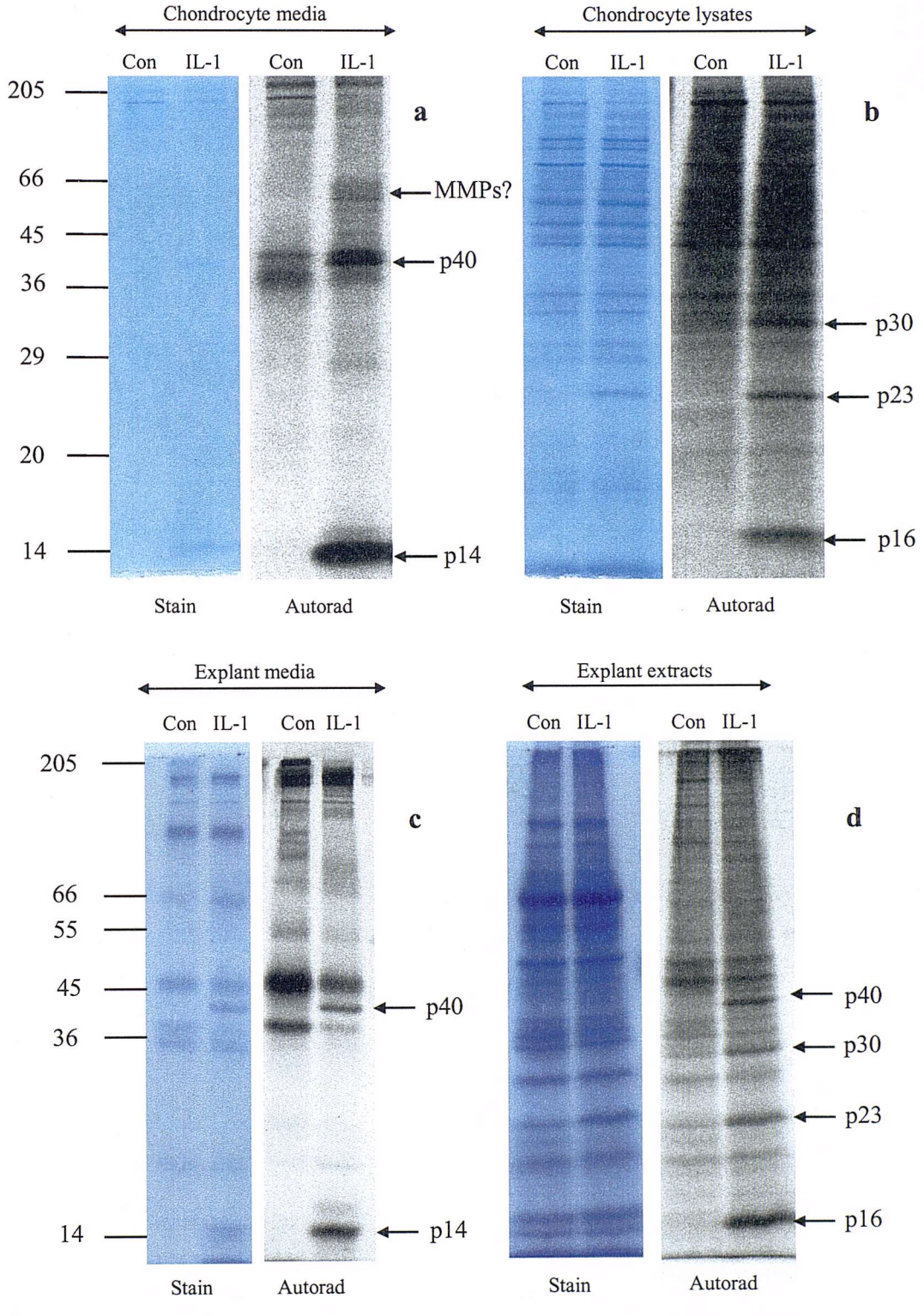


Fig 3.1. SDS-PAGE of control (Con) and IL-1 treated porcine monolayer chondrocytes (a and b) and porcine cartilage explants (c and d). Serum starved chondrocytes were treated with IL-1α (10 ng/ml) for 24 h and pulsed with [^{35}S]methionine/cysteine (50 μCi/ml) for 5 h. Explants were treated with IL-1α (100 ng/ml) and then pulsed in the same way. Medium and lysates of 6.25×10^5 cells and 50 mg cartilage were analysed by SDS-PAGE on a 12.5 % gel. Stain: Coomassie blue stained gel. Autorad: Autoradiograph.

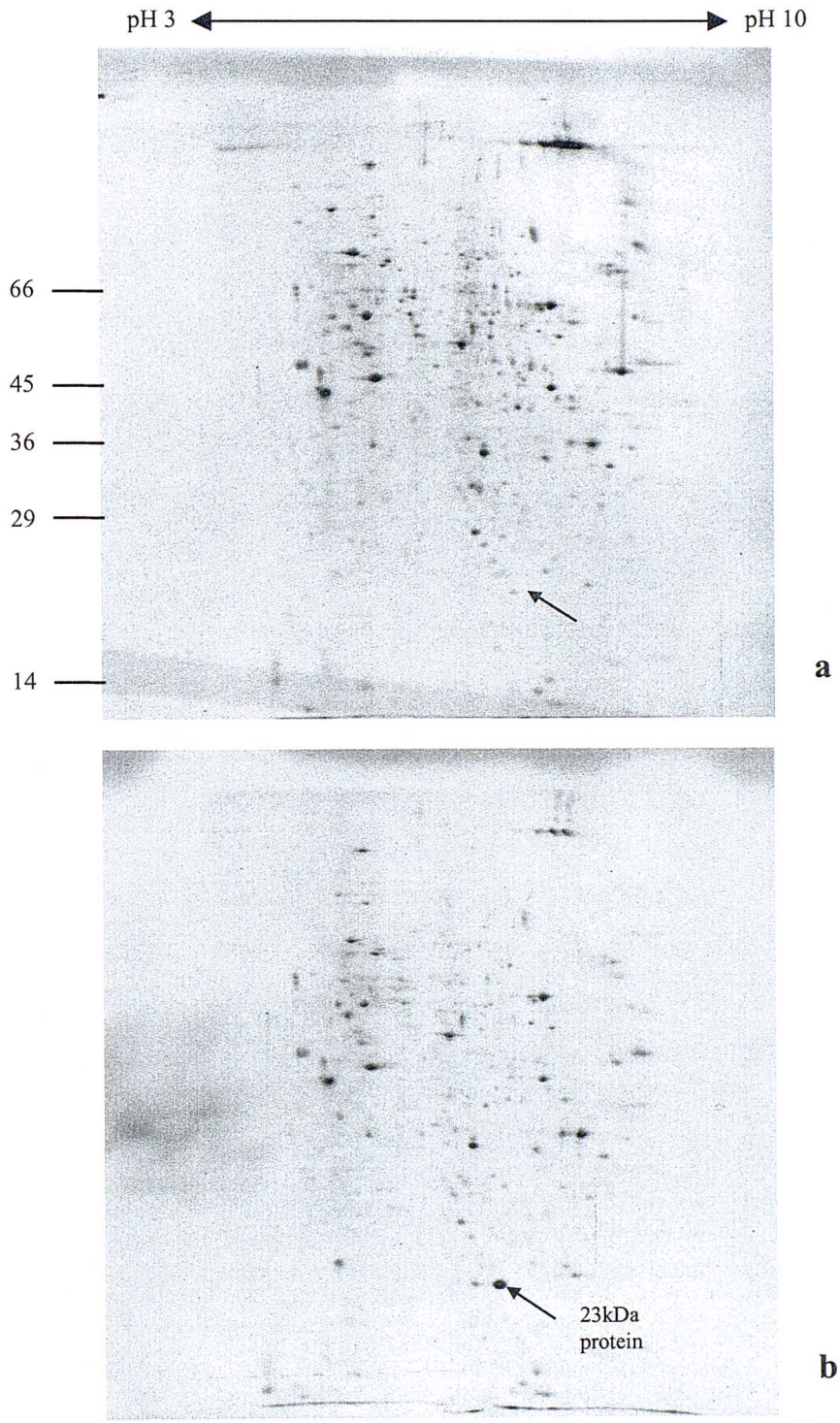


Fig 3.2. Autoradiographs of 2D-electrophoresis of lysates of control and IL-1 treated chondrocytes. Porcine articular chondrocytes in monolayer culture ($5x10^6$) were treated with vehicle (a) or IL-1α (b) for 24 h and pulsed with [^{35}S]methionine/cysteine (50 μCi/ml) for 5 h before lysis in urea lysis solution. 20 μg protein was used for isoelectric focusing on a pH 3-10 gradient. Focused proteins were electrophoresed by SDS-PAGE on a 10 % gel. Gels were stained with Coomassie brilliant blue, dried down and exposed to film for 72 h. The arrows indicate the 23 kDa protein strongly upregulated by IL-1α.

3.2.1 Identification of the 23 kDa protein as manganese superoxide dismutase (MnSOD) by mass spectrometry

The 23 kDa Coomassie Blue stained protein spot was excised from a 2D-gel of IL-1 treated chondrocytes. The protein was digested in gel with trypsin and the resulting peptides were analysed by mass spectrometry. Initially, 0.5 µl was removed from the tryptic digest supernatant and the peptide masses measured by MALDI-MS (fig 3.3). The peptide masses were compared against the theoretical digestion of the NCBI non-redundant database using Mascot (Perkins et al., 1999) software (fig 3.4). A protein with a score greater than 69 is a significant ($p<0.05$) match (fig 3.4a). No protein match was found with a score greater than 52 (fig 3.4b) so the protein was not identified by MALDI-MS.

MALDI mass spectrometry is of limited use for mammalian species such as pig which are poorly represented in protein databases compared to human and mouse. Identification of proteins of other species depends on large databases and sequence homology. Differences in amino acid sequence, which leave tryptic sites uncleaved, also affect the spectra radically.

Since we had failed to identify the protein by MALDI-MS, the remaining extracted digest was analysed by ESI MS/MS using the Q-TOF. The peptide with *m/z* = 872.5 (doubly charged) (fig 3.5a), was selected in the quadrupole and fragmented. The daughter ion spectrum of this peptide was used to deduce the amino acid sequence, AIWNVINWENVTER (fig 3.5b). The sequence was used to search the database using BLAST. The deduced amino acid sequence of the peptide matched with 100% identity a sequence in MnSOD (fig 3.6a). The E-value, a measure of the likelihood that the amino acid sequence matches a protein in a database of a given size by chance was low ($2e^{-7}$). The lower the E value is, the more unlikely it is that the match is random. Four peptides in total were sequenced by ESI MS/MS. These sequences unambiguously identified the protein as MnSOD and their location in the sequence is shown in fig 3.6b.

In the MALDI analysis, the 23 kDa MnSOD only had a probability score of 45. The MALDI peptide masses matched with MnSOD are shown in fig 3.4c, and the protein coverage of the matched peptides is shown in fig 3.4d. The reasons why MnSOD was not identified from the MALDI mass spectrum were that only 15 kDa of the porcine enzyme sequence was present in the database and there were sequence differences between pig and human (fig 3.4e). Taken together, these factors prevented retrieval of the human sequence at high probability. Subsequently MnSOD was identified from a 1D-gel of extracts of IL-1 treated cartilage explants (data not shown).

MnSOD is a protective protein that scavenges potentially toxic superoxide radicals in the mitochondria. MnSOD rapidly catalyses the specific conversion of superoxide radicals to hydrogen peroxide and oxygen ($O_2\cdot^- + O_2\cdot^- + 2H^+ \rightarrow O_2 + H_2O_2$) (Keele et al., 1970). TNF and IL-1 have both been found to induce MnSOD at the mRNA and protein levels (Wong and Goeddel, 1988). Overexpression of MnSOD has been reported to enhance cellular resistance to radiation. The protective role of TNF against radiation has been suggested to be due to the induction of MnSOD (Wong, 1995). Reactive oxygen species have been suggested to play a role in RA and can mediate hyaluronan degradation (Mapp et al., 1995).

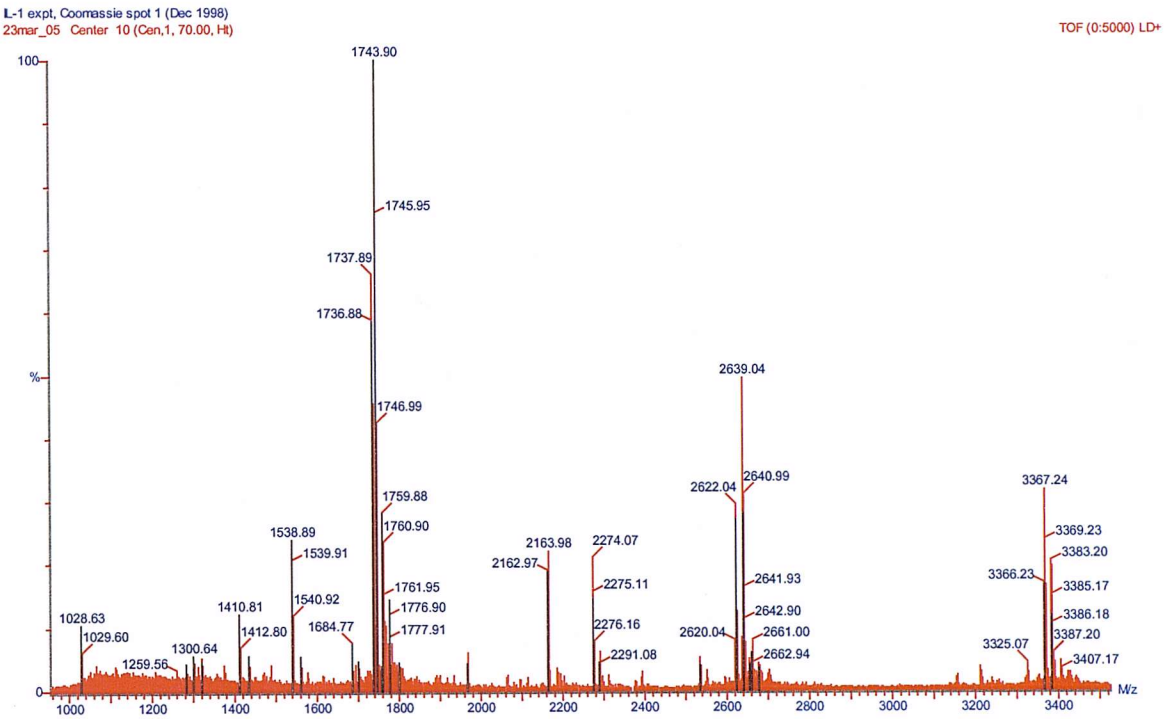
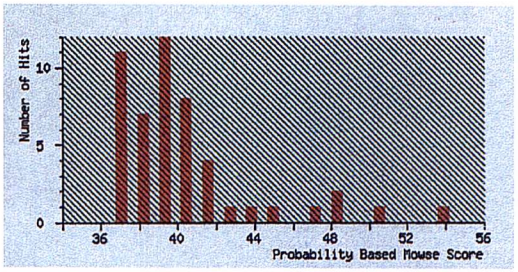


Fig 3.3. MALDI mass spectrum of tryptic peptides from the IL-1 upregulated 23 kDa protein from chondrocytes. The spot was taken from a Coomassie blue stained 2D-gel and in gel digested with bovine trypsin as described under *Material and Methods*. The spectrum shows mass-to-charge ratios (*m/z*) plotted against relative abundance. (*= Trypsin autolysis peptides).



a

b

	Accession	Mass	Score	Description
1.	gi|2570802	45435	53	(AF025381) interphotoreceptor retinoid binding prc
2.	gi|2622428	46637	50	(AE000896) conserved protein [Methanobacterium the
3.	gi|4914430	63684	48	(AL050351) SEC14-like protein [Arabidopsis thalia:
4.	gi|2498657	27945	48	NODULATION PROTEIN NOEI
5.	gi|1351139	92701	47	SUCROSE SYNTHASE ISOFORM I (SUCROSE-UDP GLUCOSYLTI
6.	gi|134677	15686	45	SUPEROXIDE DISMUTASE [MN], MITOCHONDRIAL
7.	gi|3282014	5293	44	(AJ007380) gamma-glutamyltransferase [Homo sapien:
8.	gi|3023286	36130	42	ANNEXIN V
9.	gi|2650325	7937	42	(AE001083) conserved hypothetical protein [Archae
10.	gi|2108926	7180	42	(U75019) envelope glycoprotein [Human immunodefic:
11.	gi|6093486	47745	41	STEROID HORMONE RECEPTOR FAMILY MEMBER NHR-64
12.	gi|117701	7242	41	CYTOTOXIN 3 (TOXIN V-II-3)
13.	gi|710629	20963	40	(D30762) homologue of FliY of E. coli [Bacillus s
14.	gi|2961380	56729	40	(AL022141) putative protein [Arabidopsis thaliana]
15.	gi|4741795	39324	40	(AF101047) putative dTDP-D-glucose 4,6-dehydratas

c

6. gi|134677 **Mass:** 15686 **Score:** 45
SUPEROXIDE DISMUTASE [MN], MITOCHONDRIAL

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Peptide
1028.63	1027.62	1027.60	0.02	75 -	83	1	GELLEAIKR
1410.81	1409.80	1409.79	0.02	36 -	49	0	GDVTAQVALQPALK
1538.89	1537.88	1537.88	0.00	35 -	49	1	KGDVTAQVALQPALK
2533.09	2532.09	2532.29	-0.20	95 -	118	1	LTAVSVGVQGSGWGWLGFNI

No match to: 1282.60, 1300.64, 1320.20, 1433.76, 1560.89, 1684.77, 1699.87, 1736.88,

d

```
  1 HINAQIMQLH HSEHHAAYVN NLNVVEEKYQ EALKKGDVTA QVALQPALKF
 51 NGGGHINHSI FWTNLSPNGG GEPKGELLEA IKRDFGSFEK FKEKLTAVSV
101 GVQGSGWGWL GFNKEQGRLQ IAACSNQDPL QGTTGLVPLL GIDV
```

e

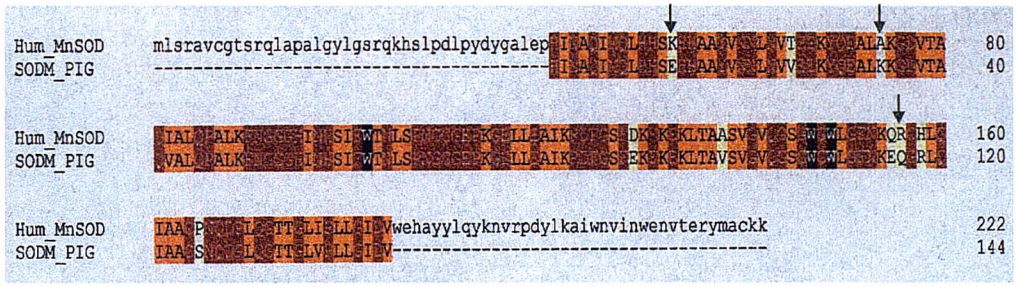


Fig 3.4. Analysis of the IL-1 upregulated 23 kDa protein spot from chondrocytes by MALDI MS. The acquired mass spectrum (fig 3.3.) was used to search the NCBI n.r. database. Proteins with a score greater than 69 are significant ($p<0.05$) for a match (a). The 15 first hits are shown in (b). The protein spot was later identified by ESI-MS as manganese superoxide dismutase (MnSOD) which with MALDI analysis only had a score of 45. The matched masses for MnSOD are listed in (c).*Observed* values are the *m/z* values of the protonated peptides from the mass spectra. *Mr(expt)* are the *m/z* values of the peptides minus the *m/z* value of the proton. The *Mr(calc)* are the theoretical peptide masses. *Delta* is the deviation of observed masses from calculated. *Start* and *End* describe were in the amino acid sequence the match is found. *Miss* indicate whether there are any missed cleavages of trypsin. The coverage of matched peptides of the MnSOD protein sequence are shown in **bold red** (d). In (e) an alignment of the 23 kDa human and 15 kDa porcine MnSOD sequence is shown. The residues are shaded from white to black using a pair-score system, with darker shades indicating residues that are generally less mutatable. Amino acids differences that affect trypsin cleavage in porcine MnSOD are arrowed.

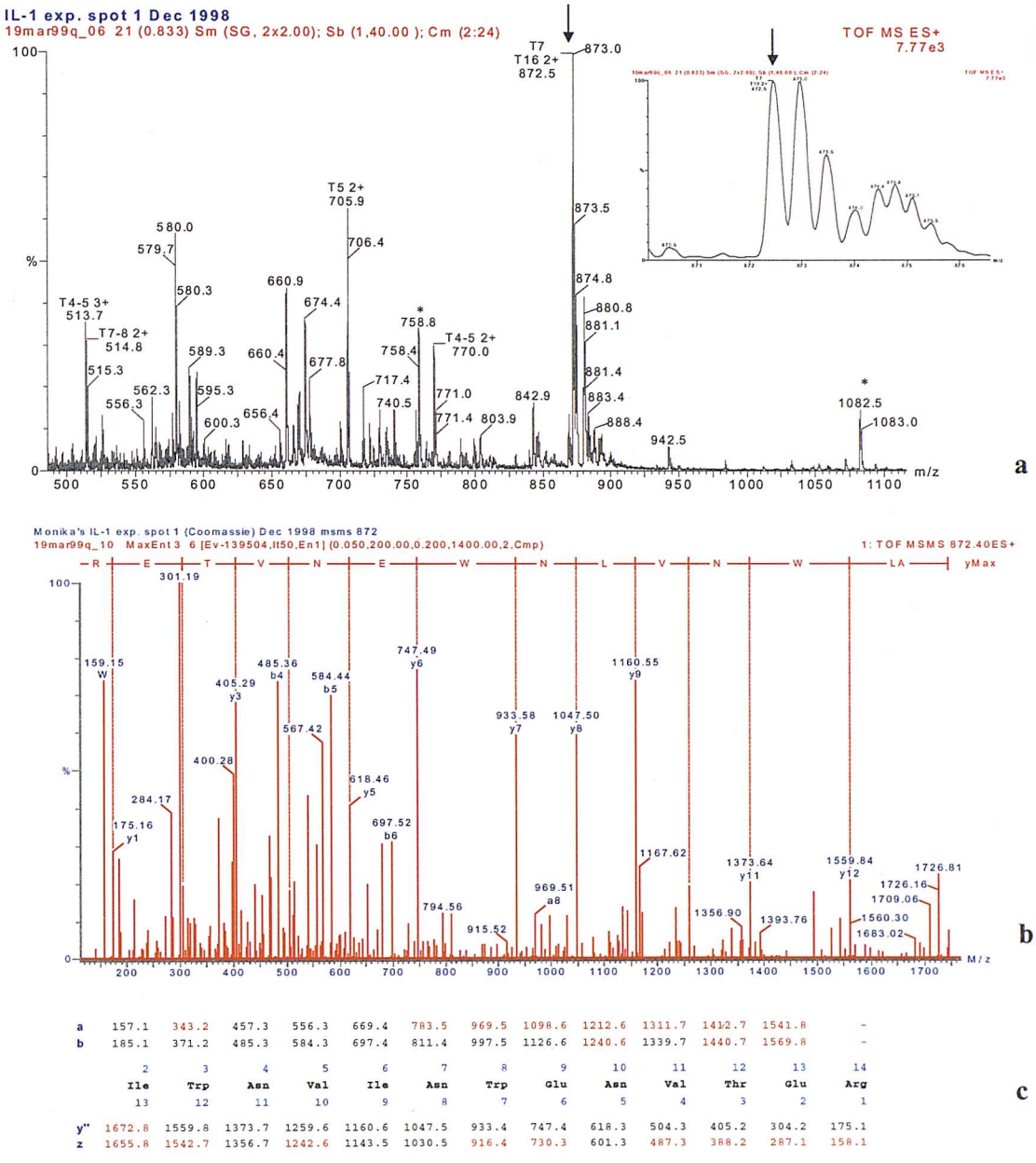


Fig 3.5. (a) Electrospray mass spectrum of the tryptic digest of the 23 kDa protein and (b) product ion spectrum of the arrowed peptide in (a). The black arrows in (a) indicate the tryptic peptide (m/z= 872.5) (2+) isolated by MS for obtaining peptide sequence. The T+ number labeled peaks in (a) indicate peptide masses that match calculated trypsin digested peptide masses from the database. (*= bovine trypsin autolysis peptides). The type of ions that have been matched (a, b, y'' or z) are indicated in black next to the amino acids in the identified peptide sequence (c).

```
                                                                  Score     E
Sequences producing significant alignments:                       (bits)  Value

pdb|1VAR|A  Chain A, Mitochondrial Manganese Superoxide Dism...    52   2e-07
emb|CAA28645.1|  (X04972) manganese superoxide dismutase [Mu...    52   2e-07
emb|CAA68533.1|  (Y00472) MnSOD precursor [Homo sapiens]           52   2e-07
sp|P04179|SODM_HUMAN  SUPEROXIDE DISMUTASE [MN], MITOCHONDRI...    52   2e-07
pdb|1AP6|A  Chain A, Tyr34->phe Mutant Of Human Mitochondria...    52   2e-07
sp|P09671|SODM_MOUSE  SUPEROXIDE DISMUTASE [MN], MITOCHONDRI...    52   2e-07
pdb|1MSD|A  Chain A, Manganese Superoxide Dismutase (E.C.1.1...    52   2e-07
emb|CAA30687.1|  (X07834) superoxide dismutase precursor (AA...    52   2e-07
emb|CAA33228.1|  (X15132) precursor [Homo sapiens] >gi|34709...    52   2e-07
pdb|1QNM|A  Chain A, Human Manganese Superoxide Dismutase Mu...    52   2e-07

 pdb|1VAR|A Chain A, Mitochondrial Manganese Superoxide Dismutase Variant With
           Ile 58 Replaced By Thr >gi|1633499|pdb|1VAR|B Chain B,
           Mitochondrial Manganese Superoxide Dismutase Variant
           With Ile 58 Replaced By Thr
           Length = 198

 Score = 52.2 bits (117), Expect = 2e-07
 Identities = 14/14 (100%), Positives = 14/14 (100%)

Query: 1   AIWNVINWENVTER 14
           AIWNVINWENVTER
Sbjct: 179 AIWNVINWENVTER 192
```

a

MANGANESE SUPEROXIDE DISMUTASE.

KHSLPDLPYDYGALEPHINAQIMQLHHSEHHAAYVNN
LNVVEEKYQEALKKGDVTAQVALQPALKFNGGGHIN
HSIFWTNLSPNGGGEPKGELLEAIKRDFGSFEKFKEK
LTAVSVGVQGSGWGWLGFNKEQGRLQIAACSNQDP
LQGTTGLVPLLGIDVWEHAYYLQYKNVRPDYLKAIWN
VINWENVTERYMACKK

b

Fig 3.6. Identification of manganese superoxide dismutase (MnSOD) by search of the NCBI non-redundant database using BLAST of the peptide AIWNVINWENVTER (a). Amino acid by amino acid is compared with the query amino acid sequence used and if all amino acids match a sequence found in the database, the identity is 100 %. The E-value is a measure of the likelihood that the amino acid sequence match the protein sequence in the database by chance. The protein sequence of the 15 kDa porcine MnSOD is shown in (b). Residues shown in red represent amino acid sequences identified by ES MS/MS.

3.3 Identification of heparin-binding glycoprotein-38

The 40 kDa protein found upregulated by IL-1 in cell medium was a Coomasssie blue stainable protein band on a 1D-gel (fig 3.1a). It was excised for trypsin digestion and analysed by MALDI (fig 3.7). The resulting peptide masses were compared against the database. The protein was identified as heparin-binding glycoprotein 38 (gp-38) with a score of 109 (fig 3.8a and b). The peptide masses matched with gp-38 are shown in fig 3.8c and the protein coverage of the matched peptides is shown in fig. 3.8d. The remaining extracted digest was analysed by ESI MS/MS. The doubly charged peptide ion with m/z= 752.9 from the acquired parent ion spectrum (fig 3.9a), was selected in the quadrupole and fragmented. The daughter ion spectrum of this peptide was used to deduce the amino acid sequence, FSNADYAVSYVLR (fig 3.9b). The sequence was used to search the database. The deduced amino acid sequence of the peptide matched with 100% identity a sequence in gp-38 (fig 3.10). The E-value of the match was $5e^{-5}$ indicating that is was unlikely to be random. Twelve peptides in total were sequenced by ESI MS/MS, and these were used to unambiguously identify the protein as gp-38. Their location in the sequence is shown in fig 3.10b. The protein was subsequently also identified from IL-1 stimulated explant medium (data not shown).

The porcine gp-38 is a homologue (84% amino acid sequence identity) of human cartilage glycoprotein-39 (gp-39) (also known as YKL-40). Synthesis of gp-39 has previously been described not to be stimulated by IL-1 or TNF-α (Nyirkos and Golds, 1990). Gp-39 mRNA is expressed in human articular cartilage and in synovium from patients with RA, but has not been detected in articular cartilage from normal subjects (Hakala et al., 1993). Its function is unknown. However, the increased expression of gp-39 in disease suggests that it may play a role in remodelling or degradation of the extracellular matrix and in the inflammatory process (Hakala et al., 1993; Shackelton et al., 1995; Verheijden et al., 1997).

The protein is predicted to be basic (calculated pI=9.17) and since basic proteins are often poorly resolved by 2D-electrophoresis, this may partly explain why it was not seen on 2D gels of cell lysates. It has a putative signal sequence, contains a

single consensus sequence for N-linked glycosylation and a heparin-binding consensus sequence. (Shackelton et al., 1995). Gp-39 is a member of the chitinase family, but does not exhibit chitinolytic activity. (Hakala et al., 1993). The human protein is secreted by chondrocytes, synovial cells, macrophages and neutrophils (Hakala et al., 1993; Kirkpatrick et al., 1997; Volck et al., 1998).

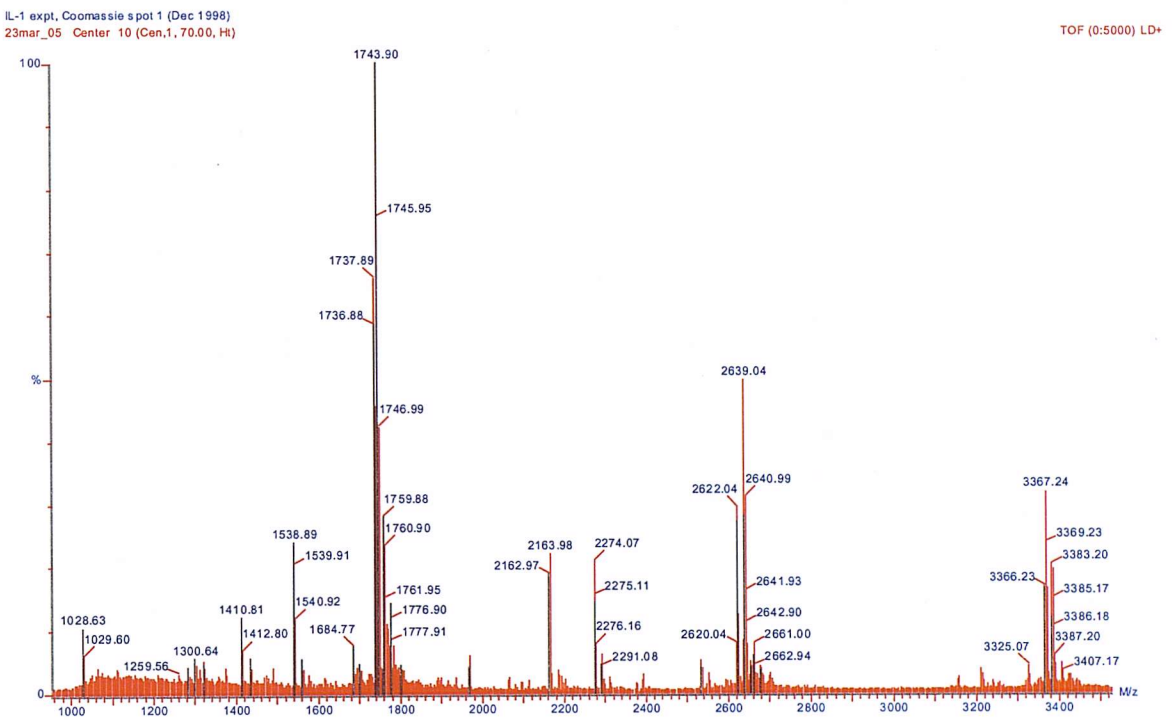
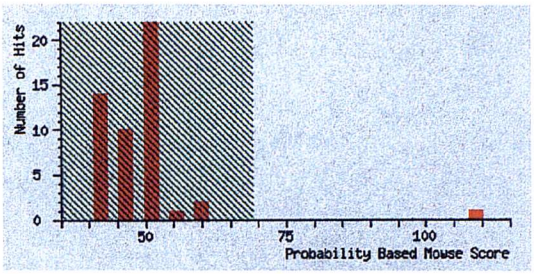


Fig 3.7. MALDI mass spectrum of tryptic peptides from the IL-1 upregulated 40 kDa protein from chondrocyte medium. The band was taken from a Coomassie blue stained 1D-gel and in gel digested with trypsin as described under *Material and Methods*. The spectrum shows mass-to-charge ratios (*m/z*) plotted against relative abundance.

a



b

	Accession	Mass	Score	Description
1.	gi|1085431	42759	109	heparin-binding glycoprotein 38K - pig
2.	gi|631129	163336	61	endosomal protein - human
3.	gi|2244807	103576	61	(Z97336) hypothetical protein [Arabidopsis thaliana]
4.	gi|2493162	28312	55	LATE EXPRESSION FACTOR 1
5.	gi|91172	49270	52	peripherin (clone 3u/5b) - mouse (fragment)
6.	gi|3914605	48217	52	RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE ACTIVASE
7.	gi|6707300	49419	52	(AF188138) unknown protein [Oxytricha nova]
8.	gi|5689255	75422	52	(AB024337) blue-light photoreceptor [Oryza sativa]
9.	gi|2500902	91290	52	SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 5A
10.	gi|1019649	87492	51	(L46816) elongin A [Rattus norvegicus]
11.	gi|1363329	87437	51	transcription elongation factor elongin A - rat
12.	gi|130256	73494	51	PUTATIVE ATP-DEPENDENT RNA HELICASE PL10
13.	gi|2688299	56788	51	(AE001145) prolyl-tRNA synthetase (proS) [Borrelia b
14.	gi|4092765	115265	51	(AF102993) kinesin related protein 2 [Nectria haemat
15.	gi|2253159	52769	51	(X59840) peripherin [Mus musculus]

c

1. gi|1085431 **Mass:** 42759 **Score:** 109
heparin-binding glycoprotein 38K - pig

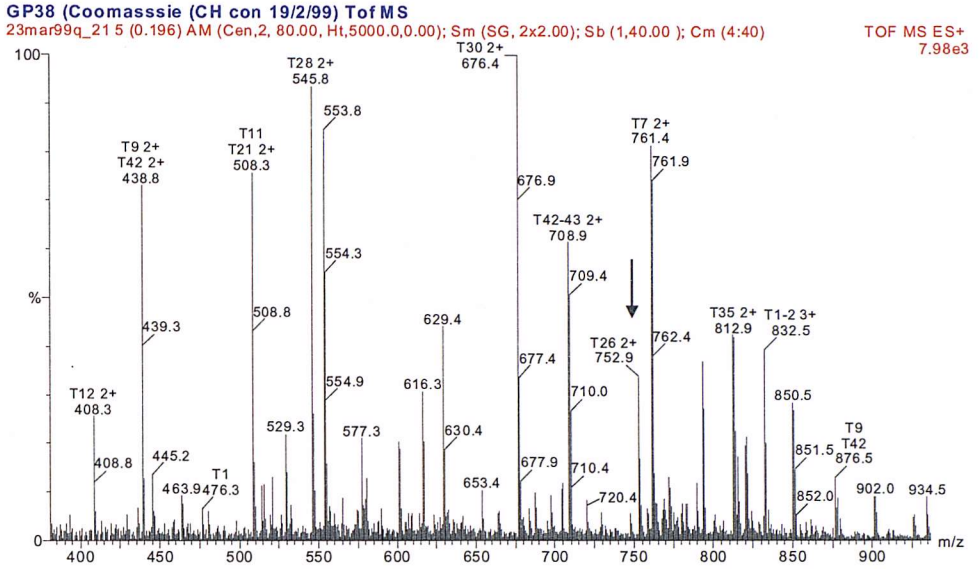
Observed	Mr(expt)	Mr(calc)	Delta	Start		End	Miss	Peptide
1090.77	1089.76	1089.60	0.16	254	-	263	0	LVMGIPTFGR
1280.78	1279.77	1279.64	0.13	214	-	224	0	QTTGHHSPLFR
1351.89	1350.88	1350.69	0.19	272	-	286	0	TDVGAPASGPGIPGR
1416.98	1415.97	1415.80	0.17	370	-	383	1	FPLTSAIKDVLAAA
1504.96	1503.95	1503.74	0.21	234	-	246	0	FSNADYAVSYVLR
1522.00	1520.99	1520.77	0.22	92	-	105	0	TLLSVGGWNFGSQR
1624.97	1623.96	1623.72	0.24	322	-	335	0	GNQWVGYDDQESVK
1867.15	1866.14	1865.85	0.29	322	-	337	1	GNQWVGYDDQESVKNK
2089.34	2088.34	2088.03	0.30	292	-	309	0	GILAYYEICDFLQGATVR
2346.49	2345.49	2345.17	0.31	290	-	309	1	EKGILAYYEICDFLQGATVR
2378.40	2377.39	2377.09	0.30	225	-	246	1	GQGDASSDRFSNADYAVSYVLR

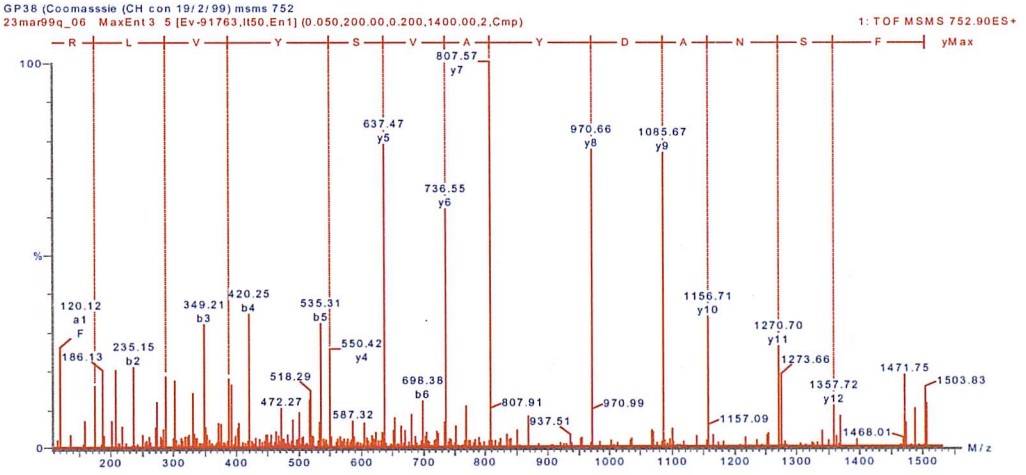
No match to: 1049.22, 1057.72, 1074.54, 1079.61, 1106.75, 1119.58, 1122.69, 1128.66, 11

d

```
  1 MGLRVAQTGF VALVLLQSCA AYKLVCYYTS WSQYREGDGS CFPDAIDPFL
 51 CTHIIYSFAN ISNNEIDTLE WNDVTLYDTL NTLKNRNPNL KTLLSVGGWN
101 FGSQRFSKIA SNTQSRRTFI KSVPPFLRTH GFDGLDLAWI SPGRRDKRHL
151 TTLVKEMKAE FVREALPGTE RLLLSGAVSA GKVAIDRGYD IAQISQHLDF
201 ISLLTYDFHG AWRQTTGHHS PLFRGQGDAS SDRFSNADYA VSYVLRLGAP
251 ANKLVMGIPT FGRSFTLASS KTDVGAPASG PGIPGRFTKE KGILAYYEIC
301 DFLQGATVRR PLGQQVPYAT KGNQWVGYDD QESVKNKAKY LKSRQLAGAM
351 VWTLDLDDFR GNFCGQNLRF PLTSAIKDVL AAA
```

Fig 3.8. Identification of heparin-binding glycoprotein 38 (gp-38) from IL-1 treated chondrocyte medium by MALDI analysis. The acquired mass spectrum (fig 3.7) was used to search the NCBI non-redundant database. Proteins with a score greater than 69 are significant ($p<0.05$) for a match. The 15 first hits are shown in (b). In (c) the matched masses for gp-38 are listed. *Observed* values are the *m/z* values of the protonated peptides from the mass spectra, *Mr(expt)* are the *m/z* values of the peptide masses minus the *m/z* value for the proton. The *Mr(calc)* are the calculated masses of trypsin cleaved peptides. *Delta* is the deviation of observed masses from calculated masses. *Start* and *End* describe were in the amino acid sequence the match is found. *Miss* indicates whether there are any missed cleavages of trypsin. The matched peptides from the protein are shown in bold red (d).





a	120.1	207.1	321.2	392.2	507.2	670.3	741.3	840.4	927.4	1090.5	1189.6	1302.6	-
b	148.1	235.1	349.2	420.2	535.2	698.3	769.3	868.4	955.4	1118.5	1217.5	1330.6	-
	1	2	3	4	5	6	7	8	9	10	11	12	13
	Phe	Ser	Asn	Ala	Asp	Tyr	Ala	Val	Ser	Tyr	Val	Leu	Arg
	13	12	11	10	9	8	7	6	5	4	3	2	1
y''	-	1357.7	1270.6	1156.6	1085.6	970.5	807.5	736.4	637.4	550.3	387.3	288.2	175.1
z	-	1340.6	1253.6	1139.6	1068.5	953.5	790.4	719.4	620.3	533.3	370.2	271.2	158.1

c

Fig 3.9. (a) Electrospray mass spectrum of the tryptic digest of the 40 kDa protein and (b) product ion spectrum of the arrowed peptide in (a). The black arrow indicates the tryptic peptide (m/z= 752.9) isolated by MS for obtaining a peptide sequence. The T+ number labeled peaks in (a) indicate peptide masses that match calculated trypsin digested peptide masses from the database. The type of ions that have been matched (a, b, y'' or z) are indicated in black next to the amino acids in the identified peptide sequence (c).

```
                                                                  Score     E
Sequences producing significant alignments:                       (bits)  Value

pir||S51327  heparin-binding glycoprotein 38K - pig >gi|6340...    44   5e-05
gb|AAB64304.1|  (AF011373) chitinase-like protein 1 [Bos tau...    41   5e-04
ref|NP_001267.1||  chitinase 3-like 1 >gi|544413|sp|P36222|C...    35   0.039
gb|AAD22610.1|  (AF062038) glycoprotein-39 precursor [Rattus...    31   0.54

 pir||S51327 heparin-binding glycoprotein 38K - pig >gi|634098|emb|CAA87764.1|
           (Z47803) 38 kDa heparin-binding glycoprotein [Sus
           scrofa] >gi|643471|gb|AAA86482.1| (U19900) 38kDa
           heparin-binding glycoprotein [Sus scrofa]
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 Score = 44.2 bits (98), Expect = 5e-05
 Identities = 13/13 (100%), Positives = 13/13 (100%)

Query: 1   FSNADYAVSYVLR 13
           FSNADYAVSYVLR
Sbjct: 234 FSNADYAVSYVLR 246
```

a

38 kDa HEPARIN BINDING GLYCOPROTEIN.

MGLRVAQTGFVALVLLQSCAAYKLVCYYTSWSQYR
EGDGSCFPDAIDPFLCTHIIYSFANISNNEIDTLEWND
VTLYDTLNTLKNRNPNLKTLLSVGGWNFGSQRFSKI
ASNTQSRRTFIKSVPPFLRTHGFDGLDLAWISPGRR
DKRHLTTLVKEMKAEFVREALPGTERLLLSGAVSAG
KVAIDRGYDIAQISQHLDFISLLTYDFHGAWRQTTGH
HSPLFRGQGDASSDRFSNADYAVSYVLRLGAPANK
LVMGIPTFGRSFTLASSKTDVGAPASGPGIPGRFTK
EKGILAYYEICDFLQGATVRRPLGQQVPYATKGNQW
VGYDDQESVKNKAKYLKSRQLAGAMVWTLDLDDF
RGNFCGQNLRFPLTSAIKDVLAAA

b

Fig 3.10. Identification of gp-38 by search of the non-redundant database using the BLAST algorithm of the identified FSNADYAVSYVLR peptide sequence (a). Amino acid by amino acid is compared with the sequence found and if all amino acids match, the identity is 100 %. The E value is a measure of the probability that the amino acid sequence match the protein sequence in the database by chance. The protein sequence of gp-38 is shown in (b). Residues shown in red represent amino acid sequences identified by ESI MS/MS.

3.4 Identification of the 16 kDa protein as a member of the serum amyloid A (SAA) family

The 16 kDa and 30 kDa proteins shown by 1D-electrophoresis to be upregulated by IL-1 in chondrocytes and explants (fig 3.1) were not seen on 2D gel electrophoresis (fig 3.2). As mentioned earlier there were various possible reasons for this failure. Since they were clearly visible on 1D-electrophoresis it was decided to fractionate the chondrocytes to simplify the protein pattern with a view to identifying them from 1D-gels.

The chondrocytes were broken mechanically by passage through a 26 guage needle and fractionated into a soluble cytosolic fraction and an insoluble membrane fraction. Autoradiography of dried 1D-electrophoresis gels demonstrated that the gp-38, MnSOD, 30 kDa and 16 kDa proteins were all particle (possibly membrane) associated (fig 3.11). Only the gp-38 was detected in the soluble fraction. The presence of the cell-associated gp-38 in the insoluble fraction was surprising since it had been shown to be a secreted protein (fig 3.1). The 16 kDa and 30 kDa proteins were not visible by Coomassie blue stain of the insoluble fraction, suggesting that they were present in low concentrations.

The insoluble fraction of chondrocytes cultured with or without IL-1 were analysed by 2D-electrophoresis and silver stained. Although the patterns were simplified with fewer spots, the 16 kDa and 30 kDa proteins were not seen (data not shown).

Therefore, ion exchange chromatography was performed in order to purify the 16 kDa and 30 kDa proteins from the particulate fraction.

Preliminary work suggested that the proteins were basic because they did not bind to an anion exchange column. A metabolically labelled insoluble fraction from 550 million cells was dissolved in 20 mM Tris buffer, pH 9.0 containing 0.1 % BRIJ 35. The supernatant was loaded onto a Resource Q anion exchange column. The material that ran through the column was collected and diluted five times with acetate buffer pH 5.0 containing 0.1% BRIJ 35. It was loaded onto a Resource S cation exchange column and eluted with a 0-1 M NaCl gradient (fig. 3.12a). Proteins were

visualised by autoradiography. A faint band in fraction 6-10 was detected, possibly corresponding to the regulated 16 kDa protein. The 30 kDa protein was not detected and was very weak in the starting material. Fractions 7-9 were most strongly labelled and were pooled and chromatographed on a Mono S cation exchange column. Using the same buffer and salt gradient as before, a faint band in fraction 4-6 was detected (fig. 3.12b). These fractions were concentrated and applied to a Superdex 75 size exclusion column with PBS containing 500 mM NaCl and 0.1 % BRIJ 35. By 1D-electrophoresis, the 16 kDa protein was detected as a strong band in the lanes corresponding to the void volume and fraction 1 (fig. 3.12d). The silver stained bands were excised and analysed by mass spectrometry (fig.3.12c).

Although good quality MALDI spectra were obtained from tryptic digests of the 16 kDa band, database searching retrieved no statistically significant matches. Serum amyloid A was indicated as a low probability candidate. The lack of statistically significant search results were not surprising since low molecular mass proteins are notoriously difficult to identify by peptide mass mapping. They usually give rise to too few tryptic peptides to give an unambiguous match. If only a homologue sequence is present in the database, then the number of matching peptides will be reduced, since any tryptic peptide containing an amino acid substitution will not contribute to the identification. The presence of more than one protein in the gel bands will further complicate the identification. Using ESI MS/MS, two peptides were sequenced from the tryptic digest of the 16 kDa band, GNYDAAQRGPGGAWAAK and ADQAANAWGR. Searching with these sequences against the NCBI and EMBL protein databases suggested that the peptides derived from members of the SAA family. The first peptide sequence was an exact match to twelve NCBI entries, most of which came from hamster or mink (E value in a BLAST search around $3e^{-16}$). Similarity to human, bovine and mouse sequences were lower. The second peptide was clearly SAA derived but differed from the human, mouse, bovine and hamster sequences by the presence of alanine rather than glutamic acid before the tryptophan. Multiple SAA genes have been described from several mammals but unfortunately not for pig. In the human, four SAA genes are encoded on chromosome 11 (Cunnane, 2001). In the mouse, five SAA family

members have been described. The mouse and human proteins were named and numbered in the order they were discovered, and therefore equivalent numbers do not necessarily refer to mouse/human homologues. Human SAA1 and SAA2 are more than 90% identical. Both are allelic; SAA1 has at least five alleles, of which three encode distinct proteins (Ducret et al., 1996). Human SAA4 (also known as C-SAA) is somewhat divergent from the other family members and is constitutively expressed, so probably can be excluded as the 16 kDa protein. In the human, SAA3 is a pseudogene because of a frameshift mutation in exon 3, which generates a downstream stop signal (Kluve-Beckerman et al., 1991). The homologous gene in the mouse, however, encodes a functioning SAA3 protein. The mouse SAA originally called SAA4 is also known to be a pseudogene. As both the protein sequences and the gene organization appear to be highly conserved it is likely that pigs similarly express multiple SAAs. But it was not possible to decide which SAA the 16 kDa protein corresponded to.

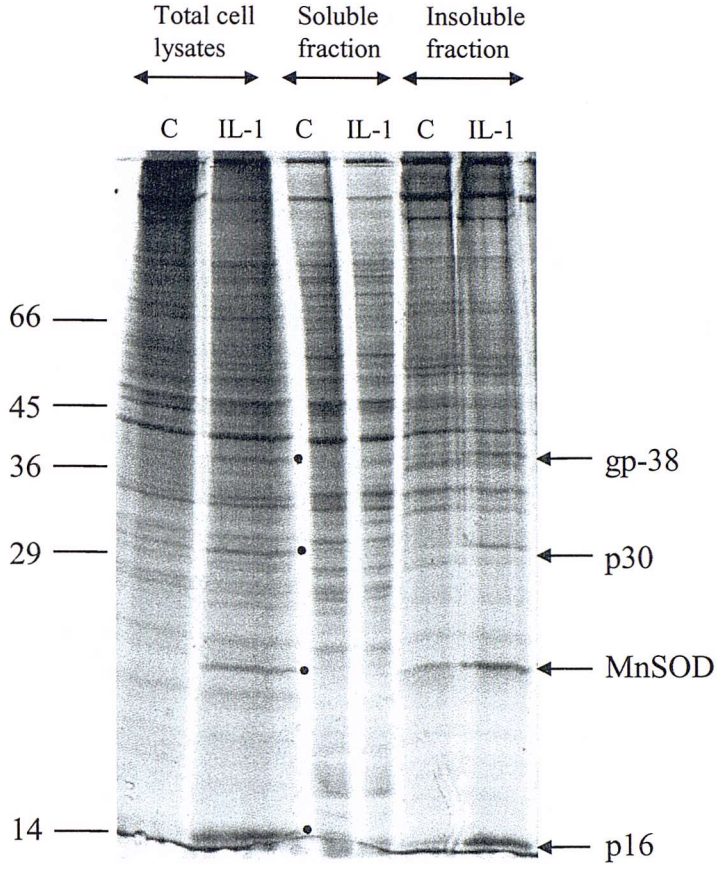
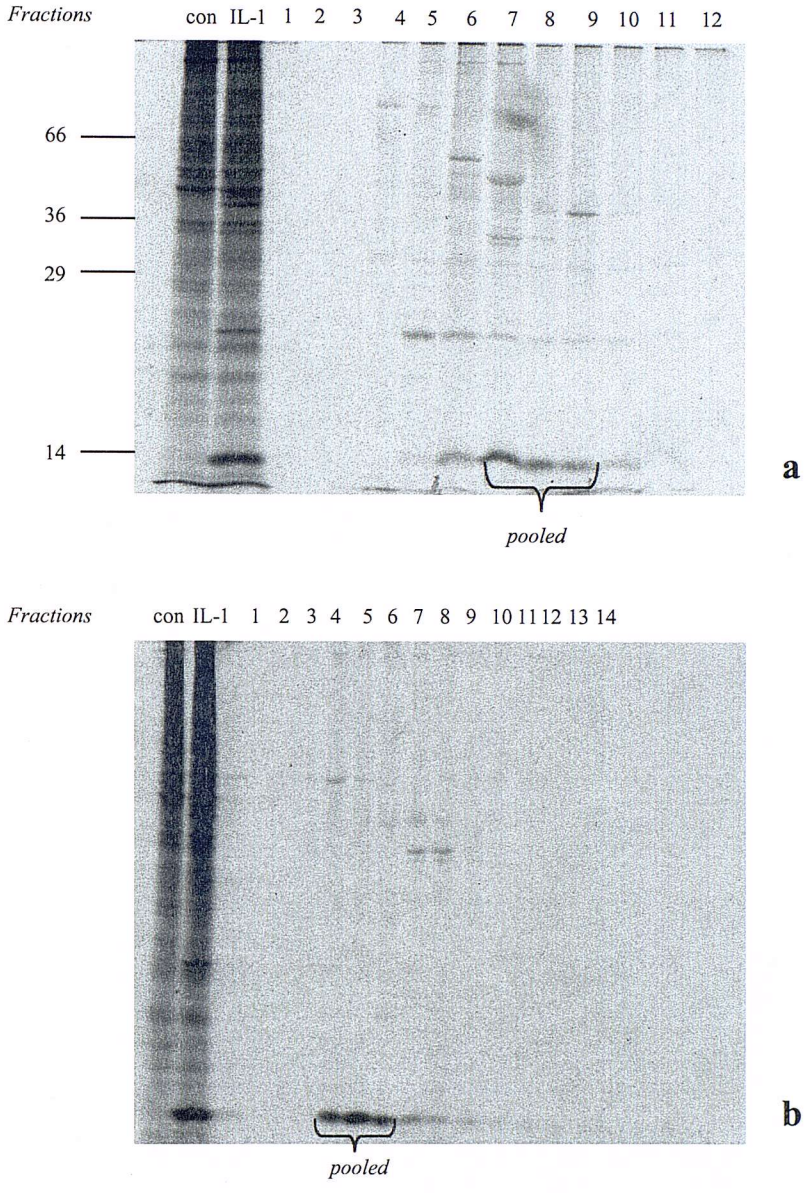
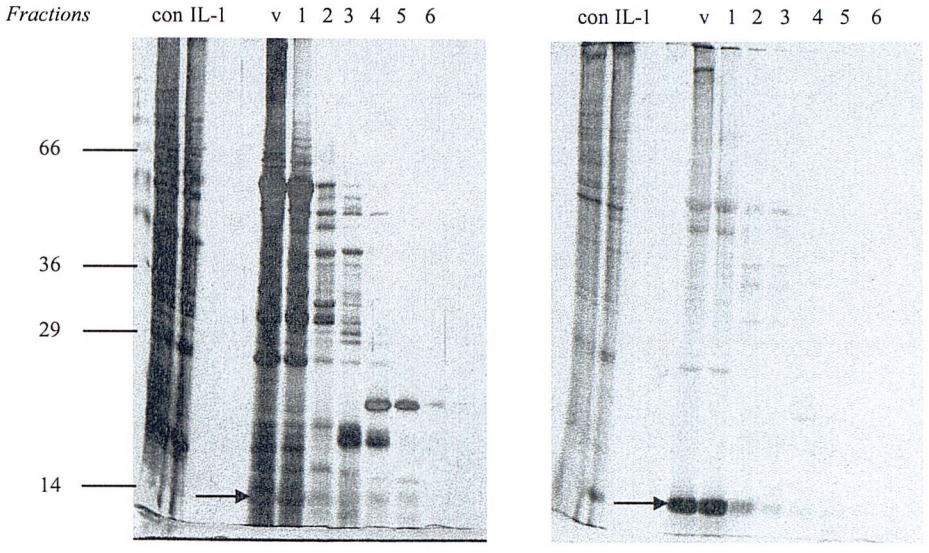


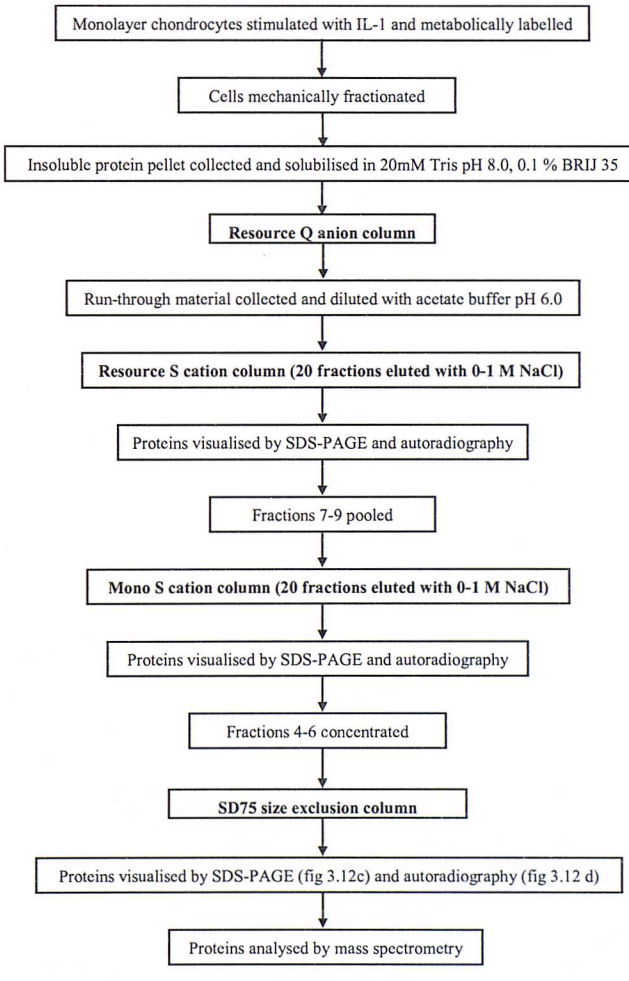
Fig 3.11. Autoradiograph of SDS-PAGE of soluble cytosolic and insoluble membrane fractions of metabolically labelled chondrocytes treated with IL-1 or vehicle (C). Porcine articular monolayer chondrocytes were pulsed with [^{35}S]methionine/cysteine for 5 h. Cells were mechanically broken using 23 guage and 26 guage needles and centrifuged at 16000 g. The supernatant, containing soluble cytosolic proteins was removed and the insoluble protein pellet was dissolved in urea lysis solution. Both fractions and total cell lysates were separated by SDS-PAGE on a 12.5 % gel, stained with Coomassie blue, dried down and exposed to film for 48 h. Black dots and arrows indicate the positions of IL-1 upregulated protein.



3.12. Purification of SAA by chromatography of the insoluble fraction of chondrocytes cultured with IL-1. A flow chart of the purification is shown in e. A metabolically labelled insoluble fraction from 550 million cells was dissolved in 20 mM Tris buffer, pH 8.0 containing 0.1 % BRIJ 35 and loaded onto a Resource Q column. The material that did not bind to the column was collected and diluted five times with acetate buffer pH 6.0 containing 0.1 % BRIJ 35. The material was applied to a 1 ml Resource S column and 20 fractions eluted with 0-1 M NaCl. Proteins were visualised by running the fractions on a 12.5 % SDS-PAGE gel, stained with silver, dried and exposed to X-ray film (a, only fractions 1-12 shown). For better resolution fraction 7-9 were pooled and loaded onto a 0.1 ml MonoS column in the SMART chromatography system. Proteins were eluted into 20 fractions using the same buffer and salt gradient. The proteins were detected with autoradiography (b, only fractions 1-14 shown). Fractions 4-6 were concentrated and applied to SD75 size exclusion column. Autoradiography detected the protein of interest in the void volume and fraction 1 (d, arrowed protein bands). The corresponding silver stained protein bands were excised and analysed by MS (c, arrowed protein bands). Starting material of the insoluble fractions of chondrocytes cultured with IL-1 or alone (con) are shown in a-d.



c d



e

3.12 continued. Purification of SAA by chromatography of the insoluble fraction of chondrocytes cultured with IL-1.

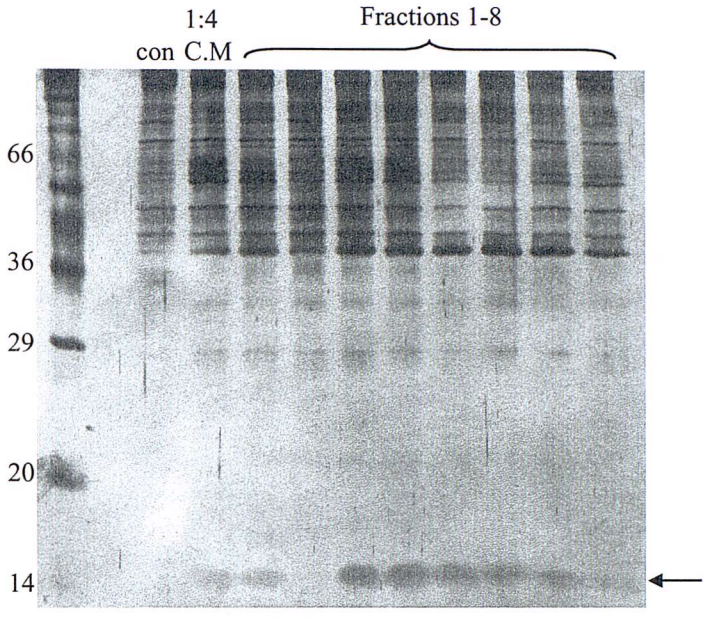
3.4.1 The secreted 14 kDa protein is a member of the SAA family

Due to the similarity in size between the cell associated 16 kDa protein and the secreted 14 kDa protein, it was possible that the latter was also a member of the SAA family. An attempt was made to identify the 14 kDa protein from cell medium after treating chondrocytes with conditioned cartilage explant medium (that also induced the production of the protein). The medium was conditioned by cutting freshly dissected cartilage into smaller pieces with a scalpel for 30 min in the medium. SDS-PAGE analysis of the cell medium was prepared by Dr Tonia Vincent at the Institute for identification of a soluble factor/s induced upon dissection of cartilage. Experiments had shown that MMP-1, MMP-3, gp-38, TIMP-1 and especially the 14 kDa protein were upregulated in monolayer chondrocytes following stimulation with conditioned explant medium. These inductions were not inhibited by IRAP, an inhibitor of IL-1 (data not shown).

Basic fibroblast growth factor was identified by Dr Vincent as one of the factors responsible for inducing the proteins (Vincent et al., 2002).

However human recombinant bFGF alone did not give as strong induction as seen with the conditioned medium treatment.

In the gel used for this analysis, protein bands around approximately 14 kDa were strongly metabolically labelled and corresponded to the silver stained bands seen (fig. 3.13). Four peptides belonging to the SAA family were identified from these bands by ESI MS/MS.



3.13. SDS-PAGE of chondrocytes treated with conditioned explant medium (CM) after Mono Q chromatography of the CM. Cartilage explants were dissected from the joint. 5 ml of medium was conditioned by cutting the cartilage in the medium into smaller pieces with a scalpel (1 g/2 ml) for 30 min. The C.M was precipitated with CPC and dialysed against 20 mM Tris/HCL pH 9.0 overnight at 4°C. The sample was applied to a Mono Q column and proteins eluted into 20 fractions with 0-1 M salt. 100 µl fractions were collected. Monolayer chondrocytes (1 million) were treated with the fractions (1/10) overnight and pulsed for 5 h the following day. Cell medium was removed and analysed by SDS-PAGE on a 12.5 % gel. The gel was stained with silver and metabolically labelled proteins were visualised by autoradiography. The position of the 14 kDa protein is arrowed. Courtesy of Dr. T. Vincent.

All identified SAA peptides are listed in table 3.1. Similar peptide sequences to those identified are present in most SAA proteins. For comparison, the protein sequences of the four known mouse SAAs (SAA1-4) are listed in fig 3.14. The peptides similar to those identified from the 14 and 16 kDa proteins are in bold. The additional data obtained from the cell medium samples were not enough to determine which SAA member/s was induced by IL-1.

SAA proteins are major acute-phase reactants. They are made mainly in the liver but have also been shown to be expressed by other cell types including chondrocytes (Vallon et al., 2001). Several proinflammatory mediators upregulate expression of SAA including IL-1, IL-6 and TNF-α. SAA proteins have been reported to play a role in chemotactic recruitment of inflammatory cells to sites of inflammation, interference with platelet function and production of MMPs. Moreover, it has been suggested that SAA protein levels in serum are elevated in RA and to a lesser extent in OA (Cunnane et al., 2000; Sipe, 1995).

Table 3.1 List of peptides identified from the 14 and 16kDa proteins.

Identified peptides	Species	Source
GNYDAAQRGPGGAWAAK	Pig	Cells
ADQAANAWGR	Pig	Cells
ADQAANAWGR	Pig	Cell medium
SDKYFHAR	Pig	Cell medium
VISDARENVQR	Pig	Cell medium
RGPGGAWWAK	Pig	Cell medium

```
                          20                  40                  60
SAA1  MKLLTSLVFCSLLLGVCHGGFFSFVHEAFQGAGDMWRAYTDMKEANWKNSDKYFHARGNYDAAQRG
SAA2  MKLLTSLVFCSLLLGVCHGGFFSFIGEAFQGAGDMWRAYTDMKEAGWKDGDKYFHARGNYDAAQRG
SAA3  MKPSIAIILCILILGVDSQRWVQFMKEAGQGSRDMWRAYSDMKKANWKNSDKYFHARGNYDAARRG
SAA4  MRLATVIVLCSLFLGVSGDGWYSFFREAVQGTWDLWRAYRDNLEANYQNADQYFYARGNYEAQQRG

                          80                  100                 120
SAA1  PGGVWAAEKISDGREAFQEFFGRGHEDTIADQEANRHGRSGKDPNYYRPPGLPDKY
SAA2  PGGVWAAEKISDARESFQEFFGRGHEDTMADQEANRHGRSGKDPNYYRPPGLPAKY
SAA3  PGGAWAAKVISDAREAVQKFTGHGAEDSRADQFANEWGRSGKDPNHFRPAGLPKRY
SAA4  SGGIWAAKIISTSRKYFQGLLNRYYFGIRNHGLETLQATQKAEEWGRSGKNPNHFRPEGLPEKF
```

Fig 3.14. Alignment of mouse serum amyloid A (SAA) proteins. Similar sequences to those identified from the 14 and 16 kDa proteins from porcine chondrocytes are in bold.

3.5 Identification of other secreted proteins induced by IL-1 in chondrocytes

Protein detection and identification from 1D-gels were improved by optimising experimental conditions. Initial gels were stained with Coomassie brilliant blue that has a detection limit of around 50 ng (for bovine serum albumin (BSA)). A silver stain method developed for mass spectrometric analysis of proteins from polyacrylamide gels (Shevchenko et al., 1996b) decreased the detection limit to approximately 5 ng (for BSA). This allowed more bands to be visualised and excised for identification. Further more, medium samples were concentrated five-fold to increase the amount of protein loaded onto the gels. More proteins were identified by 1D-electrophoresis after this. Identifications were further aided by varying the acrylamide concentration (10-12.5 %) and over-run gels to better separate the proteins.

Medium conditioned by cells in the presence of radiolabelled amino acids was prepared as before and 1 ml conditioned by approximately 5 million cells, was concentrated and run on a 10 % SDS-PAGE gel. The gel was stained with silver, dried and exposed to x-ray film. Autoradiography detected three protein bands around 60 kDa that were induced by IL-1 (fig 3.15a). These bands, seen previously in fig 3.1a, were good candidates for MMPs. MALDI and ESI MS/MS identified the upper two proteins bands as MMP-3 and the lower sharper band as MMP-1. MMPs were

also identified from concentrated IL-1-treated cartilage explant medium (data not shown). IL-1 probably also induced tissue inhibitor of metalloproteinases-1 (TIMP-1). TIMPs are important regulators of the MMPs. Collagen type XI was identified from the same digest as TIMP-1, suggesting the proteins co-migrated. However, it was clear from 2D-analysis of proteins secreted from explant medium that TIMP-1 was generally labelled (see *chapter 4*).

The high molecular mass proteins were not well resolved on the 1D-gel. In order to detect large (>70 kDa) IL-1 regulated protein, 1 ml of cell medium was separeted on a 10 % SDS-PAGE gel that was over-run so that the 36 kDa marker was at the bottom of the gel (fig. 3.15b). A regulated protein band around approximately 100 kDa was identified as complement component C3. The complement cascade is involved in inflammation reactions and cooperates with antibodies to defend vertebrates against infections. C3 is a central component of the three complement activation pathways (classical, alternative and lectin pathways. It is composed of an α -chain (110 kDa) and a β-chain (75 kDa) that are connected by a single disulphide bond. The protein is produced mainly in the liver and circulates in the blood and the extracellular fluid. It is synthesised as a proform and is activated by proteolytic cleavage. Complement factors, including C3 are readily produced in RA and OA synovial tissue (Neumann et al., 2002). C3 has previously been described to be upregulated by IL-1β and TNFα in human intestinal epithelial cells (Moon et al., 1997). The protein band detected on the gel was the C3 α-chain. The C3 β-chain was possibly detected as a faint labelled band around 75 kDa but on the stained gel, several proteins bands co-migrated and the labelled protein was not identified (fig. 3.15b).

Several prominent proteins not regulated by IL-1 were also identified (fig 3.15a and b). Fibronectin is an adhesive glycoprotein present in the matrix. Collagen type II is the most abundant collagen type in cartilage. It seems to be downregulated by IL-1 in fig 3.15b (silver stain) but this was not a consistent finding. The C-propeptide of collagen type II is the *C*-terminal part released upon maturation of collagen type II. Osteonectin (also known as *s*ecreted *p*rotein *a*cidic and *r*ich in *c*ysteine-SPARC) is an abundant matrix protein (reviewed in Lane and Sage, 1994).

Fibronectin, osteonectin, collagen type II and its C-propeptide were labelled. The other proteins identified (annexin, glia-derived nexin and phosphoglycerate kinase, see fig 3.15) from the same bands, were co-migrating proteins. Co-migrating proteins make interpretation difficult. Theoretically additional separation as obtained with 2D-gels could overcome this problem.

With a higher protein loading and increased separation it was clear that gp-38 ran as a doublet, with the lower band being the strongest. The difference in size was probably due to different glycosylation states.

In conclusion, higher protein concentration and improved separation made it possible to identify another four IL-1-induced secreted proteins.

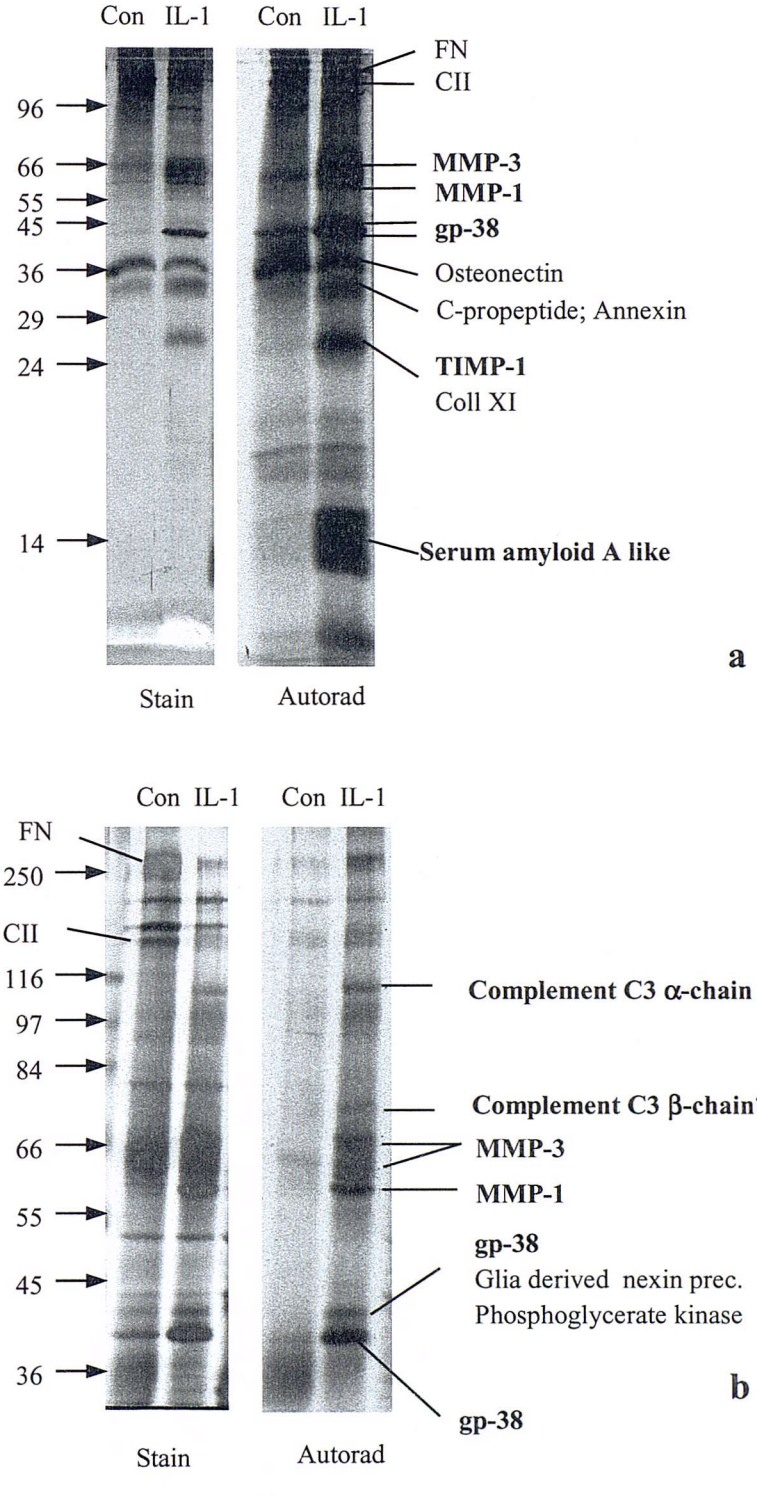


Fig 3.15. SDS-PAGE of control (Con) and IL-1 treated porcine monolayer chondrocytes. Chondrocytes were treated with IL-1α (10 ng/ml) overnight and then pulsed with [^{35}S]methionine/cysteine (50 μCi/ml) for 5 h. 1 ml of cell medium (from approximately 5 million cells) was concentrated for each sample and analysed by SDS-PAGE on a 10 % gel. The gel was run as usual (a) or until the 36 kDa size marker was at the bottom of the gel (b). The gels were stained with silver, dried and exposed to film for one week. Proteins were identified by ESI MS/MS

3.6 Study of aggrecanase activity in chondrocytes stimulated with IL-1

Aggrecan is the major proteoglycan in cartilage and provides the tissue with its ability to resist compression. The loss of aggrecan is one of the earliest changes seen in arthritic cartilage. The enzyme responsible for this degradation is thought to be aggrecanase, probably the inducible ADAMTS-4 rather than the constitutive ADAMTS-5. This enzyme, as described in chapter 1, may play a pivotal role in cartilage degradation in disease. It was disappointing but maybe not surprising given the volume of conditioned medium (<30 litres) required for its initiate purification (Hagmann, 1999; Tortorella et al., 1999), that this enzyme was not detected in either 1D- or 2D-analysis.

An attempt was therefore made to detect aggrecanase activity in chondrocytes following IL-1 stimulation. This was done by incubating conditioned cell medium with purified aggrecan. Fragments generated by aggrecanase were detected with antibodies that specifically recognise the neo-epitopes generated by aggrecanase cleavage. These aggrecanase cleavage sites are illustrated in fig 3.16.

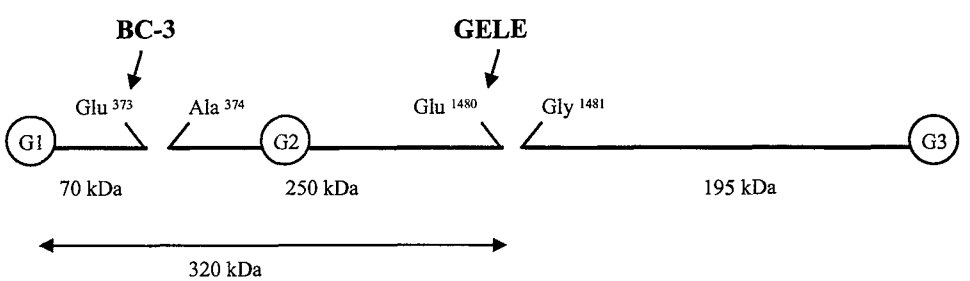


Fig 3.16. Aggrecanase-1 cleavage sites in the aggrecan core protein recognised by the BC-3 and GELE antibodies.

Cells were cultured for 40 h in the presence of IL-1 (10 ng/ml). The conditioned medium was removed and concentrated. Cell medium (equivalent to 1 million cells) was incubated with purified bovine aggrecan overnight at 37°C. Assays were made in duplicate and one sample was incubated with EDTA to control for non-metalloproteinase activity. The resulting fragments were analysed by Western blot using the BC-3 monoclonal antibody that recognises the *N*-terminal neo-epitope of the 250 kDa fragment resulting from aggrecanase specific cleavage between Glu^{373} and Ala^{374} (fig 3.17a). The experiment was repeated with the GELE polyclonal antibody that recognises the *C*-terminal of this fragment after cleavage between Glu^{1480} and Gly^{1481} (fig 3.17b). The GELE-antibody can detect the 250 kDa fragment and an additional 320 kDa fragment if aggrecan has not previously been cleaved between Glu^{373} and Ala^{374}. Using the BC-3 antibody, a 250 kDa fragment was detected in IL-1 stimulated cell medium that was not seen in the EDTA control or in unstimulated cell medium (fig 3.17a). This suggested that the aggrecan was cleaved by an IL-1 induced metalloproteinase. Analysis with the GELE-antibody confirmed this, since a doublet around approximately 250 kDa was detected in the IL-1 stimulated cell medium (fig 3.17b). No fragments were detected in the EDTA control or in unstimulated cell medium.

Aggrecanase activity is thought to be attributable to a secreted enzyme, but it has also been associated with chondrocyte membranes in bovine nasal cartilage (Billington et al., 1998). Preliminary neoepitope data from porcine chondrocytes suggested that as much as 50 % of the activity could be cell associated (data not shown). This could partly explain why aggrecanase was not detected by electrophoresis of proteins secreted from monolayer cells or cartilage explants.

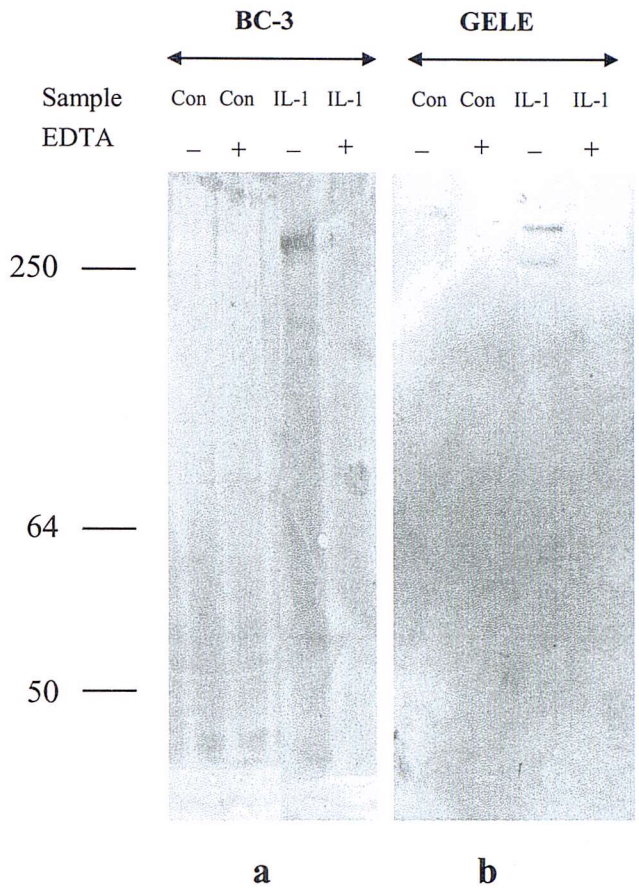


Fig 3.17. Aggrecanase neoepitope assay with BC-3 (a) and GELE (b) antibodies of chondrocyte medium treated with or without IL-1. Confluent chondrocytes were stimulated with IL-1 for 40-45 h. The conditioned medium was removed and incubated with purified bovine aggrecan overnight at 37°C. Assays were run in duplicate, with one sample incubated with EDTA to control for non-metalloproteinase activity. The reaction was terminated by addition of EDTA. Aggrecan and aggrecan fragments were deglycosylated and precipitated with acetone. Proteins were separated by SDS-PAGE on a 7 % gel, blotted onto a PVDF membrane and probed with the different antibodies.

3.6.1 Preparation of antibodies against aggrecanase-1

Having succeeded in detecting aggrecanase activity from chondrocytes stimulated with IL-1, an attempt was made to detect the protein immunologically after electrophoresis. Three antisera had been prepared against human aggrecanase-1 in sheep. They were SAK29 and SAK30, which were raised against peptides in the catalytic domain, and SAK31 against the C-terminus (fig. 3.18). The amino acid sequence of porcine aggrecanase is unknown. The amino acid sequence of the catalytic domain of porcine aggrecanase was deduced by sequencing of the PCR product following RT-PCR with human primers against porcine mRNA. This work was done by Dr Yasunobu Sawaji at the Institute. The peptide used to raise SAK29 differed in 4 out of 14 amino acids in porcine compared to human aggrecanase (fig 3.18). The sequence used for SAK30 was identical within the two species. Unfortunately the middle histidine was omitted when the peptide was synthesised for antibody production (the peptide was subsequently synthesised correctly).

It was unclear how these differences in sequence would affect the detection of porcine aggrecanase. SAK31 was not used because a positive control for full-length aggrecanase-1 was not available at the time of the experiments.

Aggrecanase-1

	SAK29 (cat.)	**SAK30 (cat.)**	**SAK31 (C-term.)**
Human	CISLNGPLSTSRHV	PVMAHVDPEEP	LEILRRRWAGRK
Porcine	C**V**GLNGP**GG**TSRHV	PVMAHVDPEEP	unknown

Fig 3.18. Peptides used for preparation of antibodies against human aggrecanase-1. The corresponding sequences in porcine are shown and amino acids that differ are in bold. The SAK29 and 30 were raised against sequences of the catalytic domain (cat.) and SAK31 was raised against the C-terminus (C-term.) of aggrecanase-1.

3.6.2 SAK29 and SAK30 tested with a deletion mutant of recombinant aggrecanase-1

The reactivity of SAK29 and SAK30 was tested against a recombinant deletion mutant of human aggrecanase-1 kindly provided by Dr Masahide Kashiwagi at the Institute. The recombinant aggrecanase was expressed in human embryonic cell line 293 using the episomal mammalian expression vector (EBNA) system. The mutant lacked the *C*-terminal thrombospondin and spacer domains. The size of the mutant was 55 kDa.

The deletion mutant was clearly detected with SAK29 but not with SAK30 (fig 3.19). This suggested that the missing histidine in the peptide used for SAK30 preparation was vital for recognition of the enzyme. SAK29 was used for further studies.

3.6.3 No aggrecanase was detected with SAK29 in porcine chondrocytes

Confluent chondrocytes were stimulated with IL-1 (20 ng/ml) for 72 h. Concentrated cell media from approximately 5 million cells were run together with the deletion mutant of aggrecanase above. Faint bands were detected around approximately 50 kDa and 100 kDa (fig 3.19). None of these were regulated by IL-1. The experiment was repeated with lysates of 1.5 million cells stimulated with or without IL-1 (20 ng/ml). More then twenty protein bands were detected with SAK29 (fig 3.20). No regulated proteins were detected. To detect the enzyme in porcine cells or cell medium, a scale-up of the material would be necessary. But the reactivity of SAK29 with porcine aggrecanase was uncertain and the SAK30 antiserum had to be re-made with the correctly synthesised peptide. Moreover, no full-length recombinant aggrecanase-1 to use as positive control was available. Because of these difficulties, the location of the enzyme on 1D- or 2D-gels was not pursued further at this stage.

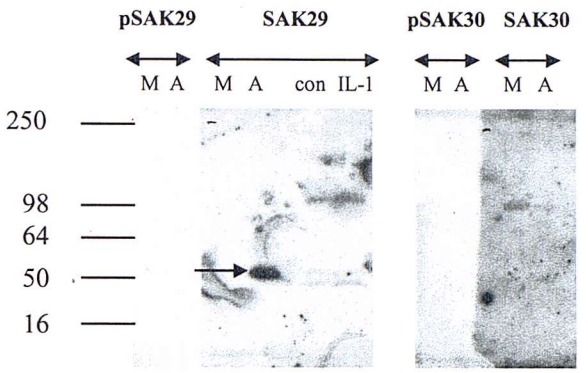


Fig 3.19. SAK29 and SAK30 antibodies tested with a deletion mutant of recombinant aggrecanase-1 (ADAM-TS4). A recombinant deletion mutant of aggrecanase-1 was analysed by SDS-PAGE on a 12.5 % gel together with cell medium from 5 million chondrocytes stimulated with or without IL-1 for 72 h. The separated proteins were blotted onto a PVDF membrane and probed with pre-immune (pSAK) and immune SAK29 and SAK30. M;mock transfer, A;aggrecanase-1 Recombinant aggrecanase protein arrowed.

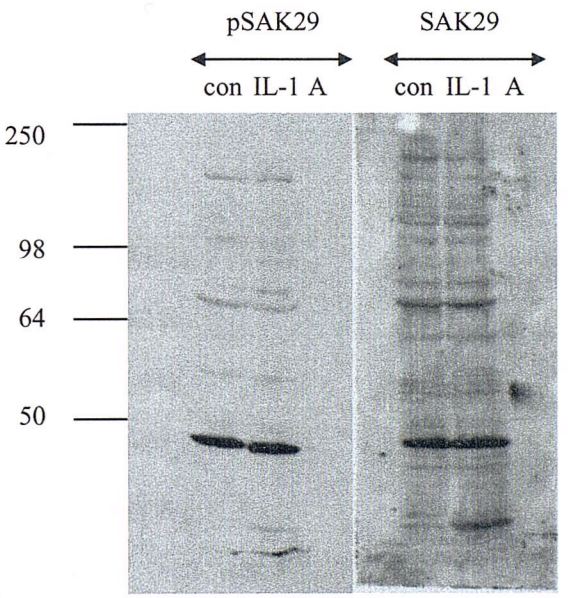


Fig 3.20. Western blot of lysates of cell treated with or without IL-1 using SAK29. Chondrocytes were stimulated with or without IL-1 for 72 h. Proteins in lysates from 1.5 million cells were separated by SDS-PAGE on a 10 % gel, blotted onto a PVDF membrane and probed with the pre-immune SAK29 (pSAK29) and SAK29.

3.7 Regulation of chondrocyte protein expression by cytokines and other factors

Chondrocytes were stimulated with various factors to study if they would have similar effect on chondrocyte protein expression as IL-1.

3.7.1 Chondrocytes stimulated with other pro-inflammatory cytokines

An experiment was set up to study if other pro-inflammatory cytokines had a similar effect to IL-1 on chondrocyte protein synthesis. Chondrocytes in monolayer were treated with IL-17 or TNFα and pulsed with [^{35}S]methionine/cysteine for 5 h. Medium samples and lysates of cells were analysed by SDS-PAGE (fig 3.21). IL-17 (100-500 ng/ml) and TNFα (100 ng/ml) induced all proteins previously seen induced by IL-1. A lower dose of IL-17 (10 ng/ml) did not induce the MMP-s or the unidentified p30 in the lysate. 10 ng/ml of TNFα had no effect on chondrocytes (data not shown). Note that the induction of complement C3 was not detected in any of the samples because the high molecular weight proteins were not sufficiently resolved.

IL-17 and TNFα were not as potent as IL-1 but could induce all IL-1 upregulated proteins.

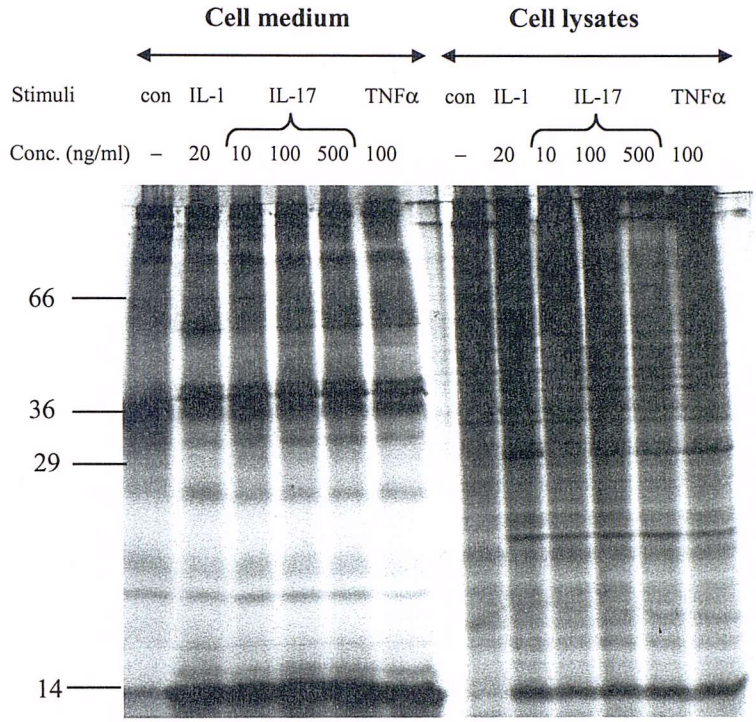


Fig 3.21. Autoradiograph of SDS-PAGE of chondrocytes stimulated with IL-17 and TNFα. Chondrocytes were treated with IL-17 (10-500 ng/ml), TNFα (100 ng/ml), IL-1 (20 ng/ml) or left untreated over night. The following day, the cells were pulsed with [^{35}S]methionine/cysteine for 5 h. Cell medium and cell lysates were analysed by SDS-PAGE on a 12.5 % gel. The gel was stained with silver, dried and exposed to film for 1 week.

3.7.2 Chondrocytes stimulated with various growth factors

Growth factors have multiple roles besides regulation of cell growth and division. To analyse the effect of growth factors on chondrocyte protein synthesis, cells were stimulated with various growth factors. Serum starved chondrocytes in monolayer were treated with bFGF, TGFβ1, epidermal growth factor (EGF), IGF-1, PDGF and VEGF. After overnight stimulation, cells were pulsed as described and medium samples and lysates analysed by SDS-PAGE (fig 3.22a). Only bFGF had a detectable effect on the pattern of chondrocyte protein synthesis. MMP-1, MMP-3, gp-38, TIMP-1 and SAA were induced with bFGF (100 ng/ml). The TIMP-1 induction was stronger than with IL-1. Complement C3, MnSOD and the unidentified p30 were not detected with bFGF stimulation. TGFβ was sometimes seen to modestly induce the C-propeptide of collagen type II (data not shown).

The lack of a visible effect by the other human growth factors could be due to poor cross-species reactivity. This was investigated by extracellularly-regulated kinase (ERK) mitogen activated protein (MAP) kinase activation in porcine chondrocytes following stimulation with the different human growth factors (except for TGFβ). All but IGF-1 induced ERK, suggesting that they were recognised by the porcine chondrocytes (fig 3.22b). The ERK assays were performed by Dr Tonia Vincent.

Stimulation of chondrocytes with BMP-2 and BMP–4 had no visible effect on gene expression (data not shown). It is not known if this was due to poor cross-species reactivity.

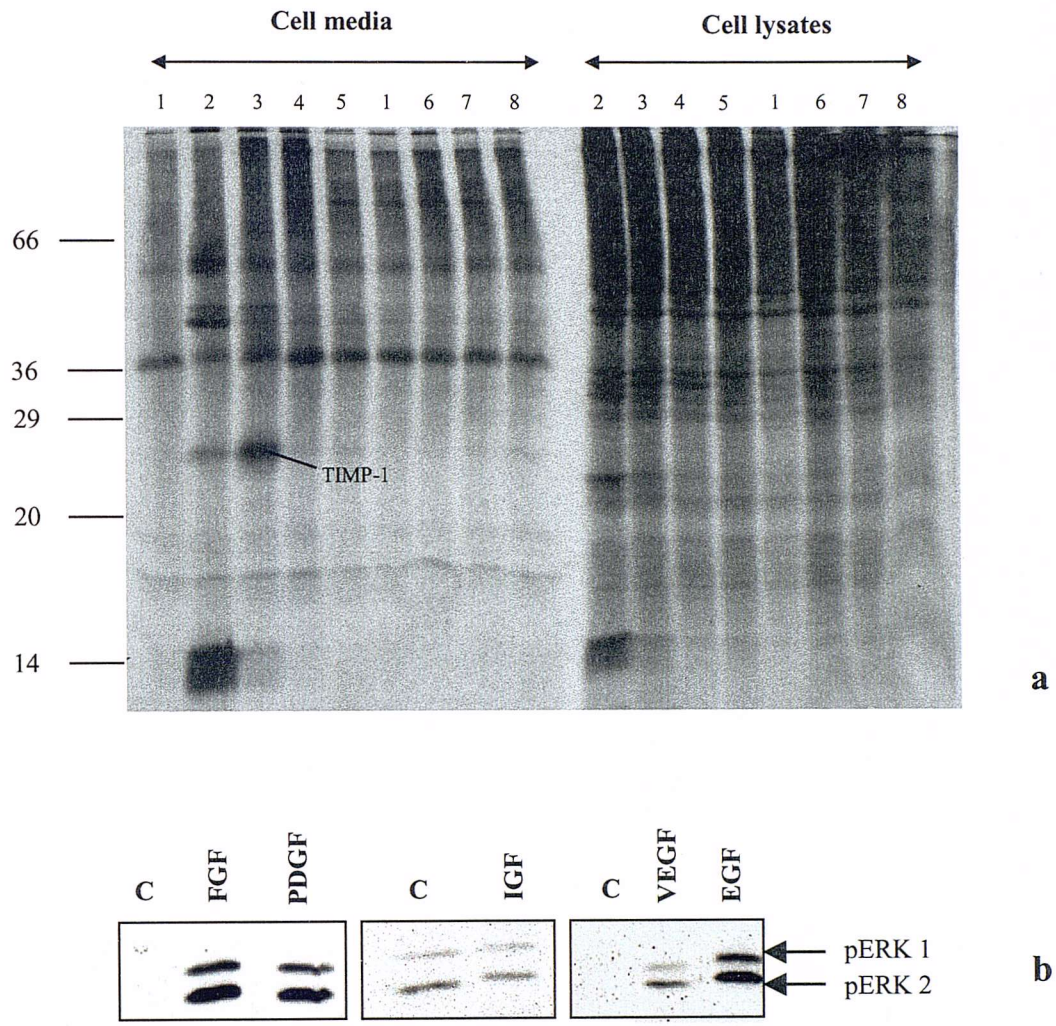


Fig 3.22. Autoradiograph of SDS-PAGE of chondrocytes stimulated with various growth factors (a). ERK MAP kinase activation of some of the growth factors are shown in (b) courtesy of Dr. T. Vincent. Primary porcine chondrocytes were treated overnight with IL-1 and growth factors as follow: **1.** Control **2.** 20 ng/ml IL-1 **3.** 100 ng/ml bFGF **4.** 20 ng/ml TGFβ **5.** 100 ng/ml EGF **6.** 100 ng/ml IGF **7.** 50 ng/ml PDGF **8.** 50 ng/ml VEGF. The following day the cells were pulsed with [^{35}S]methionine/cysteine for 5 h. Cell medium and cell lysates were analysed by SDS-PAGE on a 12.5 % gel. The gel was stained with silver, dried and exposed to film for 1 week. Proteins were identified by MALDI and ESI MS.

3.8 Human cartilage explants stimulated with IL-1

The effect of IL-1 on human chondrocyte protein expression was analysed, and the regulation of IL-1 in human and porcine chondrocytes was compared. Human normal and OA articular cartilage explants were cultured with and without IL-1 and pulsed with [^{35}S]methionine/cysteine for 5 h. Medium and explant extracts were analysed by SDS-PAGE (fig 3.23). Protein bands of interest were excised from the gel and analysed by MALDI MS and ESI MS/MS. The biosynthetic pattern of the normal and OA samples were similar. IL-1 induced MMP-1 and MMP-3 in both samples. They were detected in both the medium and the extracts. In contrast to porcine cartilage, YKL-40/gp-39 was downregulated by IL-1. Another member of the chitinase-family, YKL-39 was strongly labelled in the medium. This protein had not been detected in porcine material. In contrast to YKL-40, it does not bind to a heparin column, and is apparently not glycosylated (Hu et al., 1996). No labelled protein corresponding to SAA was detected in the human samples. Protein bands that could correspond to complement C3 (fig 3.23 marked with question marks) were detected but they were not identified. MnSOD was upregulated by IL-1 in the explant extracts but the unidentified p30 was not detected. TIMP-1 was detected as a labelled protein band in both control and IL-1 stimulated samples. It was not strongly induced by IL-1 as it had been with porcine chondrocytes.

It was clear from the experiment that the effects of IL-1 differed between human and porcine articular cartilage. Further studies of proteins secreted from human cartilage explants were performed using 2D-electrophoresis (see *chapter 5*).

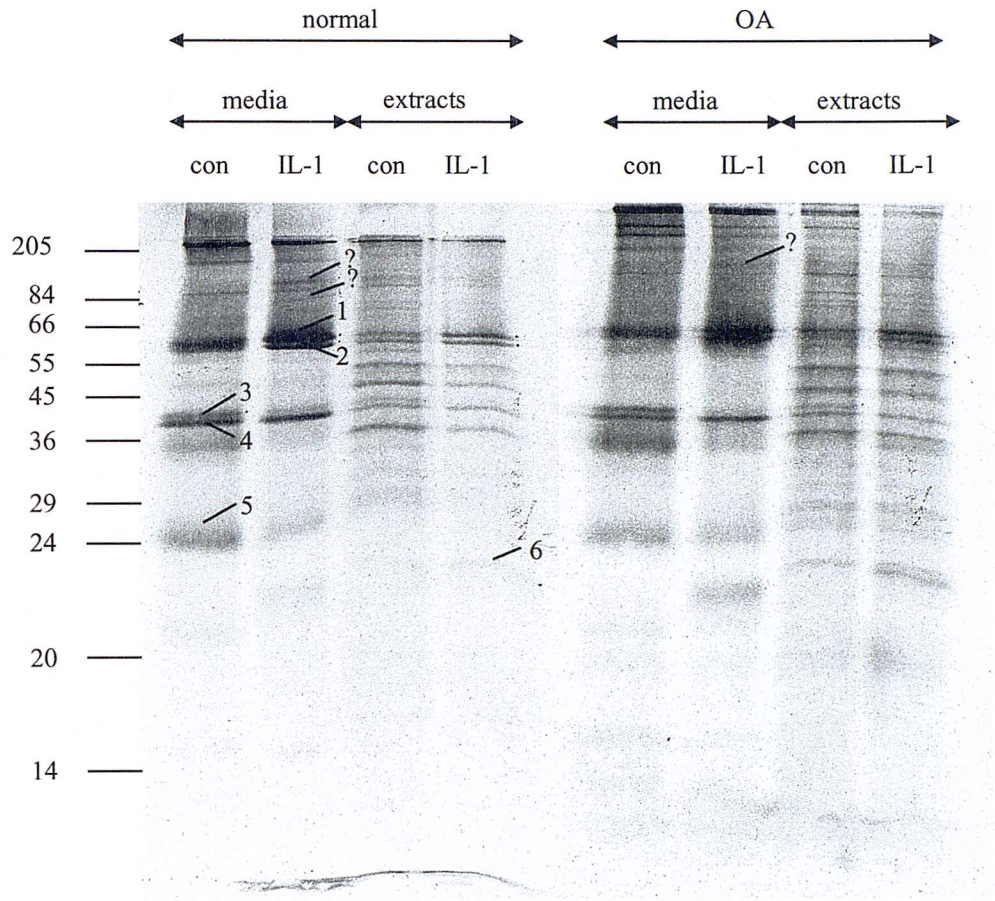


Fig 3.23. Autoradiograph of SDS-PAGE analysis of normal and OA human cartilage explants treated with IL-1 or vehicle (con). Normal articular cartilage was obtained from a 67 year old man post mortem and OA articular cartilage came from the hip of a 78 year old man after replacement surgery. Cartilage explants were cultured with or without IL-1 (100 ng/ml) over night and the pulsed with [^{35}S]methionine/cysteine for 5 h. Medium was removed and the explants extracted with dissociative lysis buffer for 45 min at 4°C. Medium and explant extracts were analysed by SDS-PAGE on a 12.5 % gel. The gel was stained with Coomassie brilliant blue, dried and exposed to x-ray film for 4 days. Proteins were identified by MALDI and ESI MS.
1. MMP-1, **2.** MMP-1/MMP-3, **3.** YKL-40/gp-39, 4. YKL-39, **5.** TIMP-1, **6.** MnSOD

3.9 Summary of results

Chondrocyte protein expression and its regulation by IL-1 was studied by 1D- and 2D-electrophoresis and mass spectrometry. Seven proteins were identified that were upregulated by IL-1 in monolayer chondrocytes. These were MnSOD, SAA, TIMP-1, gp-38, MMP-1, MMP-3 and complement component C3. MnSOD is cell associated and the others are secreted proteins. All but TIMP-1 and complement C3 were also identified from cartilage explants. TIMP-1 was later identified by 2D-electrophoresis of cartilage explants (see *chapter 4*). The scarcity of human material necessitated the use of porcine tissue in these experiments. This created some problems regarding the identification of proteins by MALDI MS, because there are relatively few porcine sequences in the databases. Identification of proteins across species is often difficult unless the proteins are very highly conserved. Therefore identification of the animal proteins were generally made by sequencing peptides by ESI MS/MS. The experiments also showed that the environment of the chondrocytes affected the pattern of proteins secreted and this is explored further in the next chapter. The proteins responding to IL-1 were not so environmentally dependent, however there were very marked differences in the porcine and human genes responding to the cytokine. Thus gp-38 was markedly induced in porcine cells, but YKL-40/gp39 was constitutively expressed and inhibited by IL-1 in human chondrocytes. SAA was prominently induced in porcine but was not seen in human.

1D-electrophoresis and mass spectrometry are powerful tools for the separation and identification of proteins. However, many proteins secreted by chondrocytes such as for instance, cartilage oligomeric matrix protein, chondroadherin and various proteoglycans were not detected. Some protein bands were found to contain several co-migrating proteins on 1D. In order to overcome these problems and identify more proteins, especially regulated proteins, 2D-electrophoresis needed to be performed.

In the following chapter I will describe how methods were developed to study proteins secreted by chondrocytes in cartilage explants and in monolayer cultures by 2D-electrophoresis.

Chapter 4

2D-Electrophoresis of Proteins Secreted by Chondrocytes

Part I 2D-electrophoresis of proteins secreted from cartilage explants

4 Introduction

Initial analysis of proteins secreted by chondrocytes using 1D-electrophoresis, showed that the protein expression pattern of unstimulated cells varied with their physiological surroundings. Despite this, cells cultured as either cartilage explants or in monolayer showed a very similar pattern of protein induction upon IL-1 stimulation. About seven secreted proteins were identified as being up-regulated by stimulation with IL-1. A limitation of the analysis by 1D-electrophoresis was that bands cut out from the gels sometimes contained more than one protein. Because of this, and the expectations of resolving more proteins, 2D-electrophoresis was performed. It seemed a realistic task for proteomic analysis to identify all the proteins secreted by chondrocytes, since this meant resolving and analysing perhaps one or two hundred protein spots.

Preparing 2D-gels of proteins secreted by chondrocytes turned out to be more difficult than expected. The proteins did not isoelectric focus. This was found to be due to the polyanionic molecules present in the conditioned medium.

This chapter describes how techniques were developed to overcome the isoelectric focusing problem and resolve proteins secreted by chondrocytes in both cartilage explants and monolayer culture.

4.1 2D-electrophoresis of cartilage explant medium

Proteins secreted into explant medium failed to isoelectric focus (fig 4.1). This was likely to be due to the presence of proteoglycans particularly aggrecan, whose negatively charged glycosaminoglycan chains (fig 1.1) would bind to proteins at low ionic strength and interfere with their isoelectric focusing. The GAG content in the sample shown in fig 4.1 was estimated to be 50 μg/ml.

It was therefore necessary to find a way of removing the proteoglycans or their glycosaminoglycan chains, from the samples. The glycosaminoglycan chains could be cleaved with chondroitinases, or the proteoglycans could be removed by precipitation.

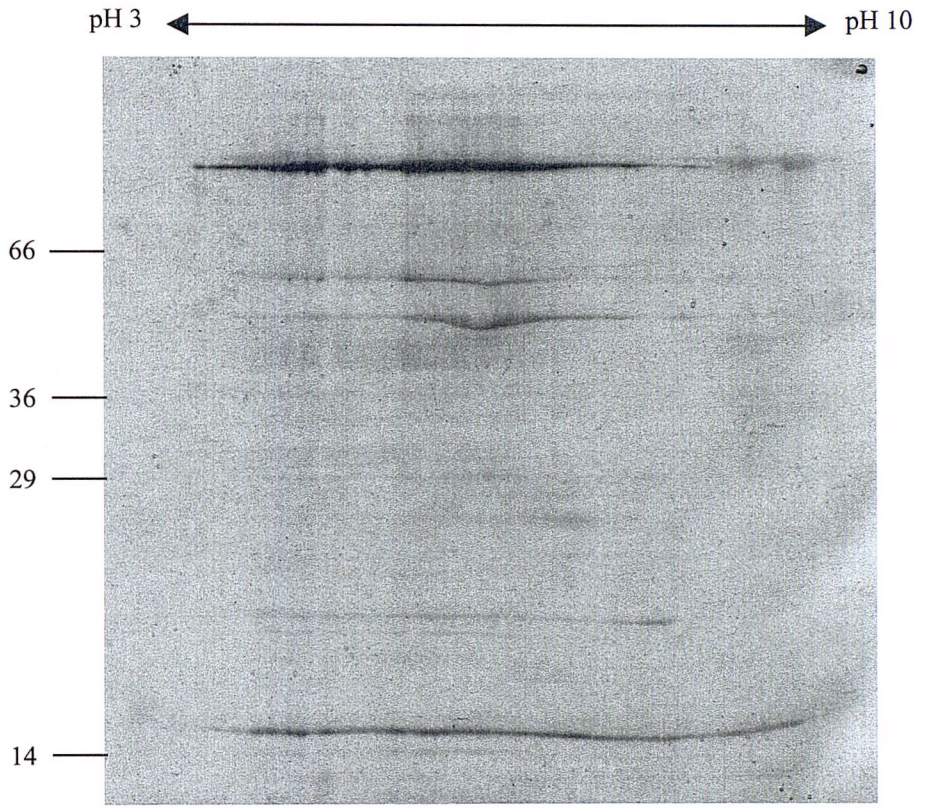


Fig 4.1. 2D-electrophoresis of explant medium. 30 g of porcine articular cartilage was cultured for 24 h in serum-free medium. The medium was replaced with fresh medium and the cartilage cultured for a further 5 h. Explant medium was removed and its GAG content estimated. The sample was dialysed against water and lyophilized. Proteins were isoelectrically focused on a pH 3-10 gradient, followed by SDS-PAGE on a 12.5 % gel. The gel was stained with Coomassie brilliant blue.

4.2 Digestion of GAG chains in explant medium by chondroitinase ABC lyase

A standard method of using chondroitinase ABC lyase to remove GAG chains (Yamagata et al., 1968) was tried. This enzyme removes chondroitin sulphate and dermatan sulphate side chains of proteoglycans, yielding core proteins with enzymatically modified linkage oligosaccharides. The enzyme is highly specific for the GAG chains and does not affect the protein core or keratan sulphate chains. Keratanases can be used to remove keratan sulphate chains, however they were found unnecessary in the following experiments to remove GAG chains.

After investigating different concentrations of chondroitinase ABC lyase, it was found that 0.0025 u of chondroitinase per 10 μg GAG for 1h removed 90 % or more of the GAG from the explant medium samples. The remaining GAG content was estimated with the dimethyl methylene blue (DMB) assay.

2D-electrophoresis was performed on IL-1 treated explant medium. The resulting 2D-gels were well focused but contained a large amount of bovine serum albumin (BSA), which was used by the supplier as a carrier for the enzyme (fig 4.2a). The enzyme preparation was also run alone and contained approximately a hundred different proteins detectable by silver staining (fig 4.2b). Chondroitinase is approximately 80 kDa and the presumed enzyme position is arrowed. A larger amount of the enzyme preparation (0.625 u) was used for 2D-electrophoresis than was used for digestion of GAG in the explant medium sample (0.1 u). Chondroitinases without added BSA from Seikagaku and Sigma were also analysed by 1D-electrophoresis. Both contained a similarly high degree of impurities as that with the albumin carrier shown in fig 4.2b. These preparations were thus judged unsuitable for use in 2D analysis of complex mixtures of proteins by MS.

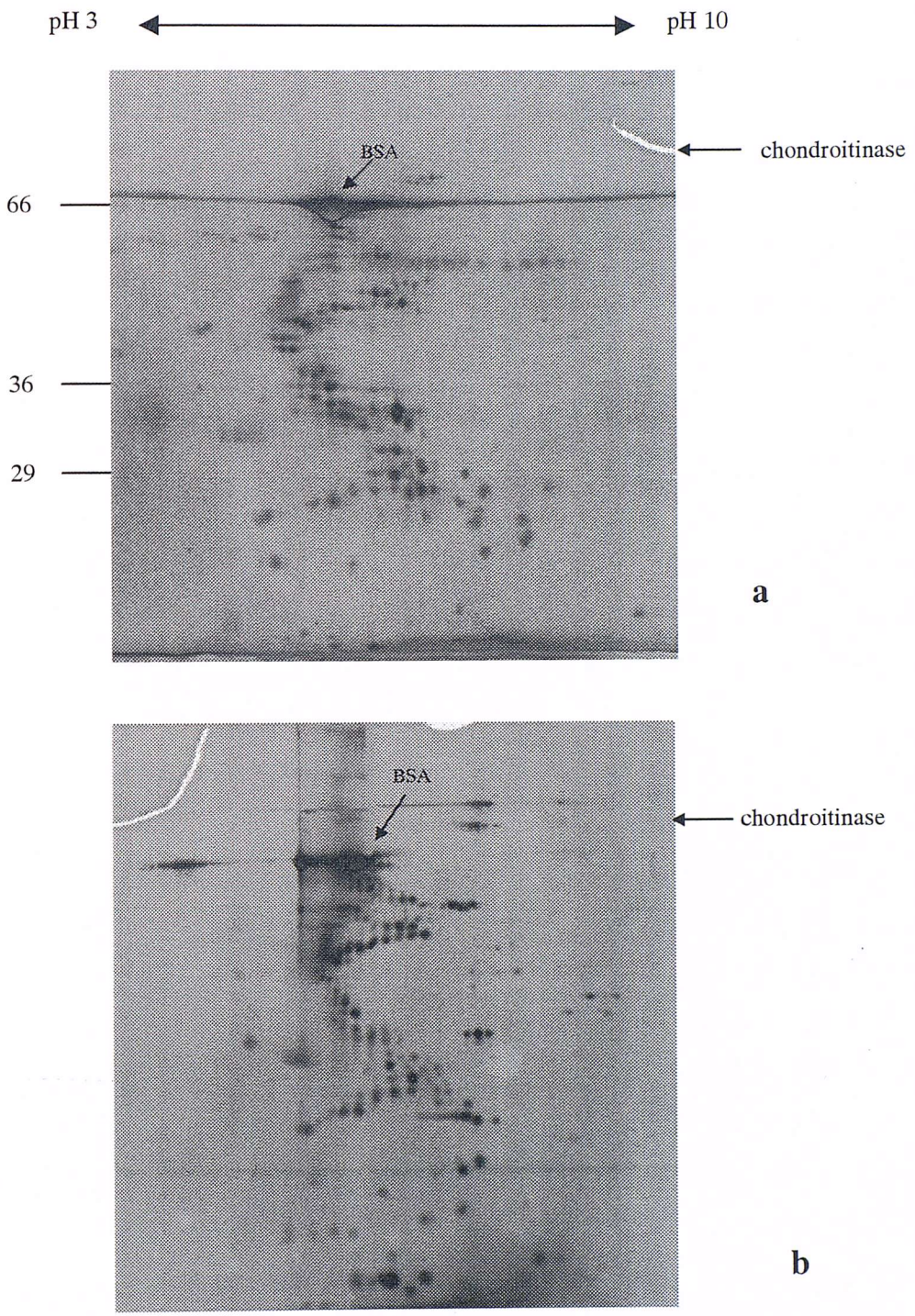


Fig 4.2. 2D-electrophoresis of explant medium treated with chondroitinase ABC lyase (a) and the enzyme preparation electrophoresed alone (b). (a) 2 ml of explant medium was treated with 0.1 u of chondroitinase for 1 h at 37°C. The medium was dialysed against water at 4°C and lyophilized. The protein sample was dissolved in urea lysis solution and loaded onto a pH 3-10 gradient gel strip for first dimension focusing. SDS-PAGE was performed on a 12.5 % gel. (b) 0.625 u of the enzyme preparation containing BSA as a carrier (Seikagaku), was mixed with urea lysis solution (without protease inhibitors present) for 2D-analysis as described. Gels were silver stained. The presumed enzyme position (80 kDa) is arrowed, as is the spot for BSA.

4.3 Use of cetylpyridinium chloride (CPC) to remove GAG from explant conditioned medium

The high content of impurities in the chondroitinase preparations led to the conclusion that it would be too difficult to determine whether a 2D-gel spot originated from cartilage or from the enzyme preparation.

Therefore, it was decided to remove the proteoglycans by precipitation. Cetylpyridinium chloride (CPC) (systemic name: hexadecylpyridinium chloride) is a cationic detergent with the empirical formula $C_{21}H_{38}ClN$. It is commonly used for proteoglycan purification (Laurent and Scott, 1964) and as a dental plaque formation preventative. It has not to my knowledge previously been used for removal of proteoglycans to study remaining proteins in a complex mixture. CPC forms micelles at room temperature and interacts with the polyanionic GAG chains of proteoglycans. The positively charged pyridinium group binds to and precipitate out the highly negatively charged GAG chains. Unreacted CPC can be crystallised out below its critical micelle temperature (20°C).

Adjusting the critical electrolyte concentration (CEC) controls the precipitation of polyanions. The higher the salt concentration, the fewer polyanions are precipitated. CPC precipitates polyanions in the sequence keratan sulphate, chondroitin sulphate, polynucleotides, hyaluronan, and acidic glycoproteins in descending order of CEC. For chondroitin sulphate the CEC is about 0.9 M (Prof. John Scott, personal communication).

Two mg CPC/mg proteoglycan, plus 3 mg/ml excess was added to control and IL-1 treated explant medium. This is the amount added to purify proteoglycans and was recommended by Prof J.E. Scott (University of Manchester) who developed the technique (Laurent and Scott, 1964). The proteoglycan content was estimated as GAG with the DMB assay. The GAG content varied in explant medium samples between 100-400 µg/ml but was normally around 200 µg/ml. Usually IL-1 treated samples contained 10-20 % more GAG. Generally, following Scott's method meant that about 15 mg CPC/mg GAG was added. The proteoglycan-cetylpyridinium complex precipitated immediately at room temperature as white flakes. After 5 min, the sample was cooled on ice for one hour to crystallise out excess CPC. The supernatant was

removed and the pellet was washed with 0.5 M sodium acetate in 70 % ethanol to remove the CPC. 1D-electrophoresis and silver staining of the CPC precipitated explant medium and the proteoglycan containing pellet showed that there were losses of proteins from the supernatant after CPC precipitation (fig 4.3).

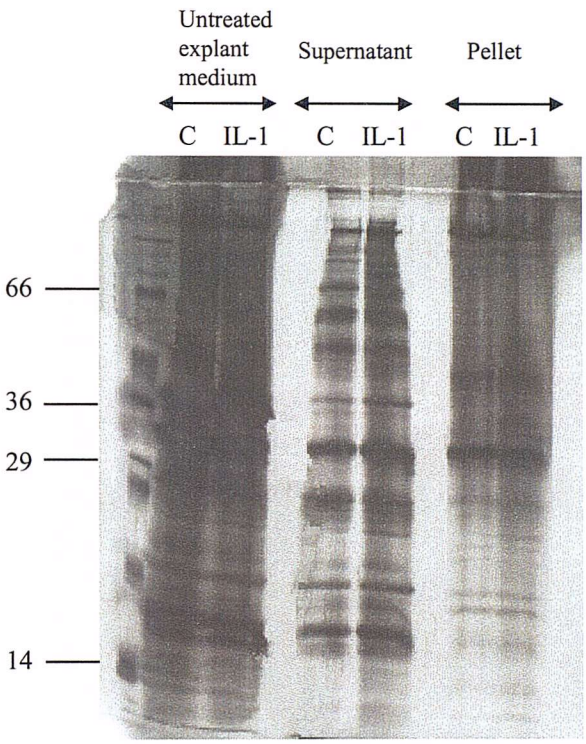


Fig 4.3. SDS-PAGE showing control (C) and IL-1 treated explant medium before and after CPC precipitation. 3 g of cartilage explants were treated with or without IL-1 for 24 h and then pulsed with [^{35}S]methionine/cysteine (50 µCi/ml) for 5 h. Explant medium was removed and precipitated at room temperature with 2 mg CPC/mg GAG plus 3 mg CPC/ml of total volume, corresponding to 10.5 mg CPC/mg GAG for the control and 12.7 mg CPC/mg GAG for the IL-1 treated sample. Excess CPC was precipitated out on ice. Supernatants were dialysed into water and lyophilized. The proteoglycan rich pellet was washed with 0.5 M sodium acetate in 70 % ethanol to remove the complex-bound CPC. Samples were analysed by SDS-PAGE on a 12.5 % gel. The gel was silver stained.

Repeated experiments with CPC aroused concerns that the protein recovery in the supernatant after CPC precipitation was variable and often low. Furthermore, proteins not detected in the supernatant were not always found in the pellet, suggesting that some proteins were non-specifically pulled down with the CPC-proteoglycan complex and then lost in the wash for removal of CPC. The often large loss of proteins from the supernatant indicated that they were interacting with the excess CPC and precipitated out with it. The effect could be limited either by reducing the CPC concentration used, or by avoiding the cold precipitation of the excess CPC. However, if the precipitation of excess CPC was omitted, a large amount of CPC in the sample could be difficult to remove through dialysis, and subsequently could interfere with isoelectric focusing of proteins.

4.3.1 CPC precipitation of proteins is concentration and temperature dependent

An experiment was set up to study how protein recovery and successful removal of GAG were dependent on the CPC concentration and precipitation temperature. IL-1 treated explant medium was obtained as before. The GAG concentration was estimated and the medium sample divided in aliquots, which were treated with increasing amounts of CPC from 0.8 to 48.8 mg/mg GAG. Each sample was divided in two, half being cooled on ice to precipitate out excess CPC, while the rest was directly prepared for ID-electrophoresis (fig 4.4). The final GAG concentration was measured and plotted against CPC concentration (fig 4.5). The gels showed that when excess CPC was precipitated on ice, increasing CPC concentration resulted in protein precipitation, with loss of a number of proteins from the supernatant. Consequently, more proteins were detained in the pellets (fig 4.4b). Analysis of the GAG concentration after CPC precipitation showed that 5.7 mg CPC/mg GAG was sufficient to remove GAG to a level of less than 10 μg/ml (fig 4.5b). However, at this concentration protein losses from the supernatant became very apparent.

If the cold precipitation of excess CPC was omitted however, no major protein losses were observed with any CPC concentration (fig 4.4a), and 2.4 mg CPC/mg GAG was enough to remove a sufficient amount of GAG (fig 4.5a). This indicated that it was the cold precipitation that was responsible for non-specific losses of protein. The removal of GAG was more efficient with precipitation at room temperature when compared with precipitation at room temperature followed by cold precipitation. This suggested that proteins were binding to crystalline CPC. The GAG-CPC complex formation could be partly inhibited by these other interactions thus the need for a higher concentration of CPC for complete removal of GAG by the cold precipitation.

Some proteins though, may be lost via a specific interaction with proteoglycans, as for example link protein. The large size of the proteoglycans prevents them from entering the separation gel during electrophoresis, instead they can be detected as a smear in the stacker. A protein found strongly associated with the proteoglycan-containing pellet was separated by 1D-electrophoresis, in gel digested and analysed by MS. The protein was identified as cartilage link protein and is seen as a strong protein band above the 36 kDa marker in the pellets in fig 4.4a.

Several proteins were precipitated along with the proteoglycans at 2.4 mg CPC/mg GAG at room temperature (fig 4.4a), but no longer precipitated with increased CPC concentration. Since the aim was to use as little CPC as possible (because it has to be removed prior to isoelectric focusing), the protein precipitation at low CPC concentration was studied further. Assuming that these proteins were interacting with proteoglycans ionically, it might be possible to prevent this by increasing the salt concentration. The precipitation at room temperature was therefore repeated at increased salt concentration. Samples were precipitated with 0.8-24.4 mg CPC/mg GAG in the presence of 306 mM NaCl in the medium compared with 158 mM. The protein precipitation at low CPC concentration still occurred (fig 4.6), but GAG removal was less efficient with increased salt concentration (fig 4.7).

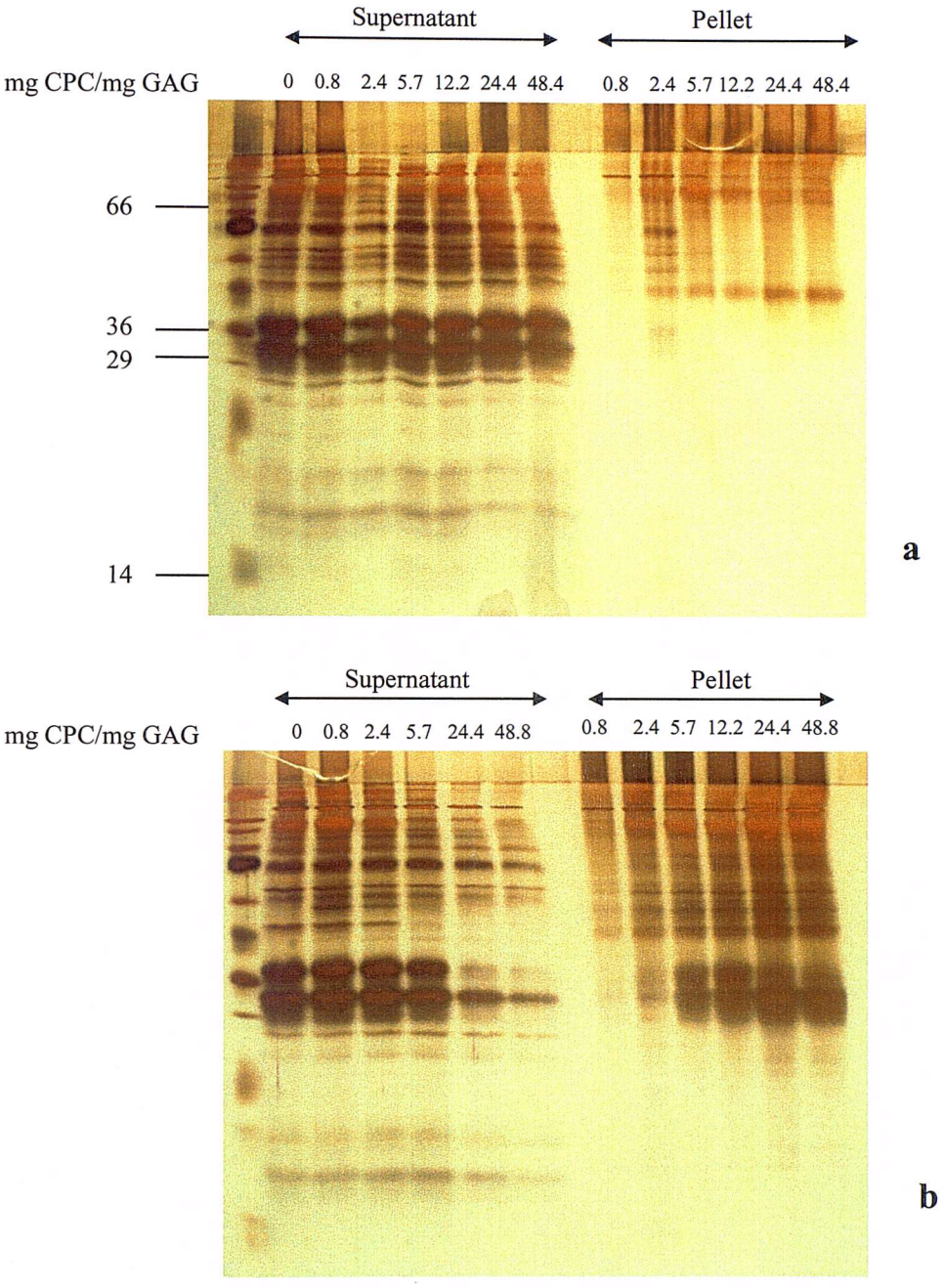
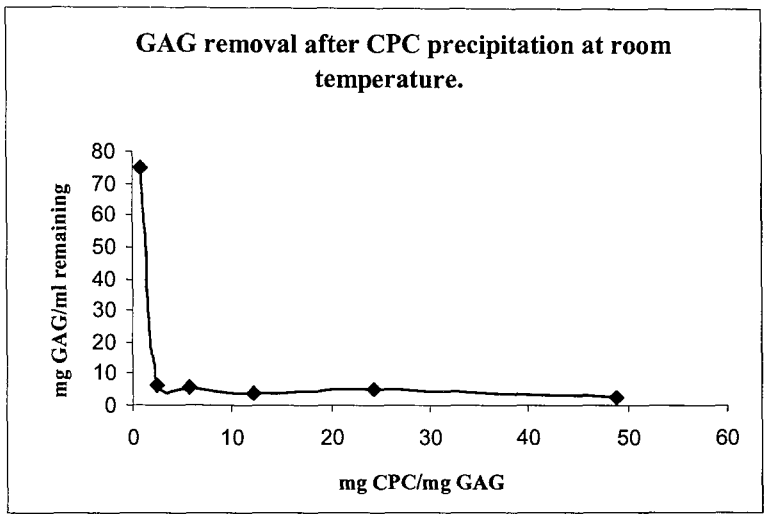
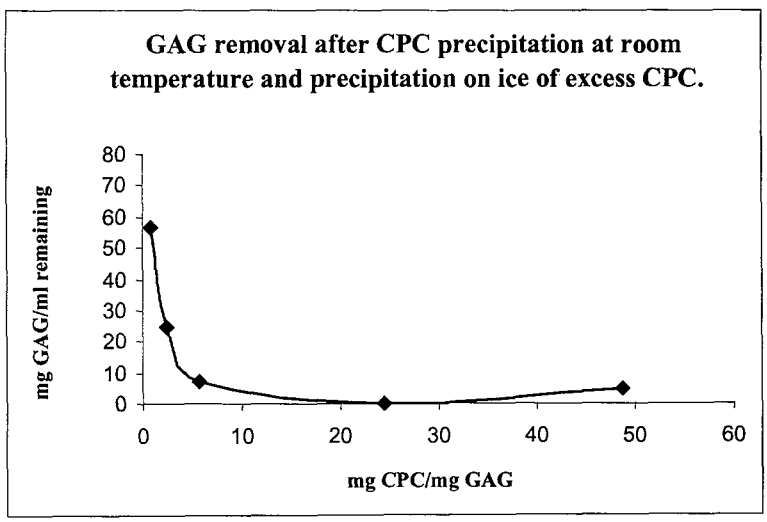


Fig 4.4. SDS-PAGE showing the effect of CPC concentration upon precipitation of proteins from explant medium at room temperature (a). In (b), the sample has been treated as in (a) and then on ice for precipitation of excess CPC. Cartilage explants were treated with IL-1α (100 ng/ml) for 24 h. The medium was replaced with fresh medium and the cartilage cultured for a further 5 h. Explant medium was removed and its GAG content estimated. 400 µl/sample was precipitated in room temperature with 0-48.8 mg CPC/mg GAG. Two samples for each concentration were made. For each concentration one sample was precipitated for 30 min at room temperature (a) and one sample was precipitated at room temperature for 30 min followed by 1 h on ice to precipitate out excess CPC (b). The proteoglycan rich pellets were treated with 0.4 M sodium acetate in 90 % ethanol to remove the complex bound CPC. Supernatants and pellets were analysed by SDS-PAGE on a 12.5 % gel. Gels were silver stained. The 12.2 mg CPC/mg GAG precipitated sample is missing in (b) due to a technical problem.



a



b

Fig 4.5. GAG remaining after CPC precipitation of explant medium at room temperature (a), and after CPC precipitation followed by precipitation of excess CPC on ice (b). After CPC precipitation with 0-48.8 mg CPC/mg GAG, the residual GAG in the supernatant was analysed with the DMB assay. The 12.2 mg CPC/mg GAG sample is missing in (b) due to technical problems.

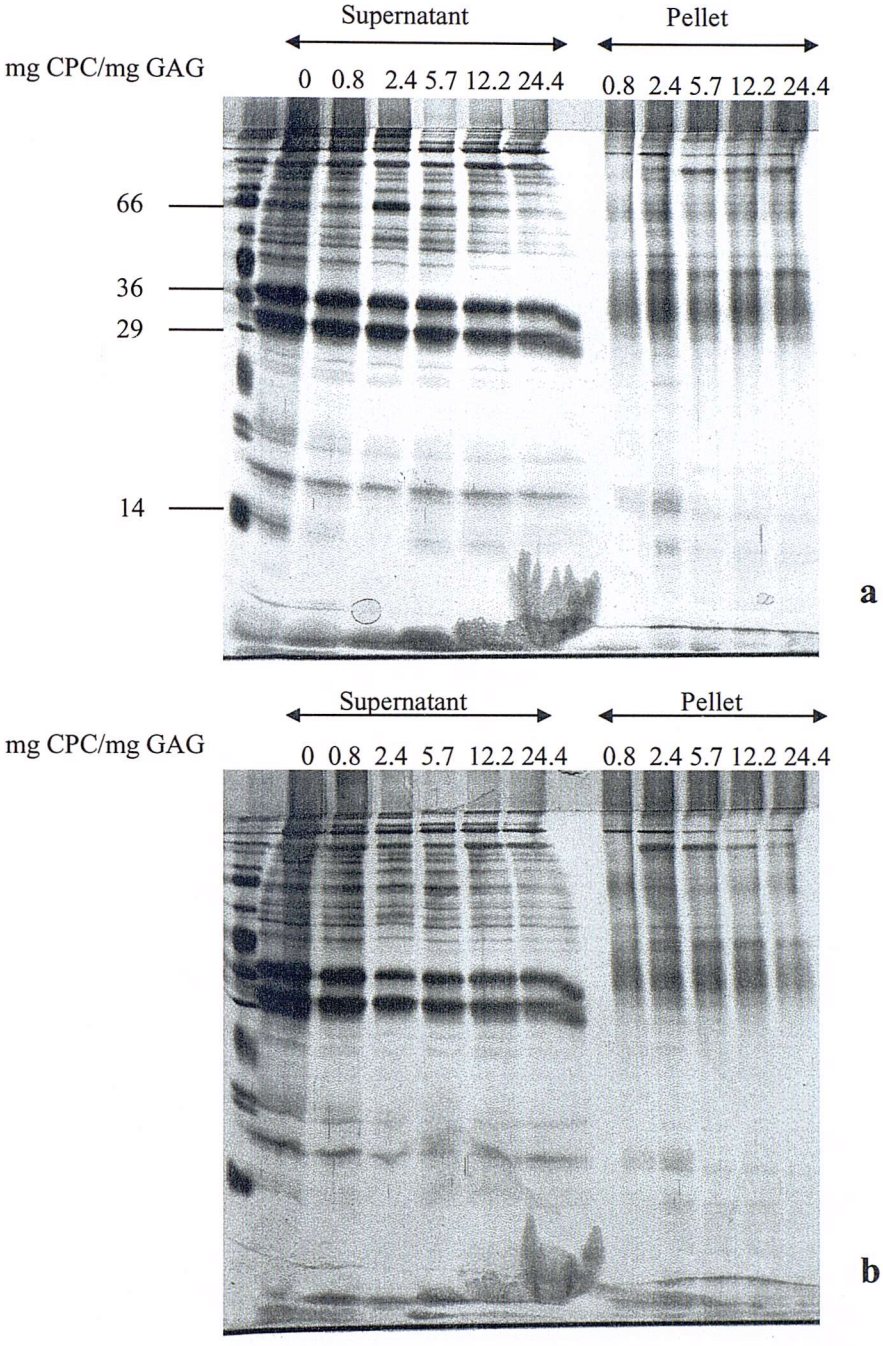
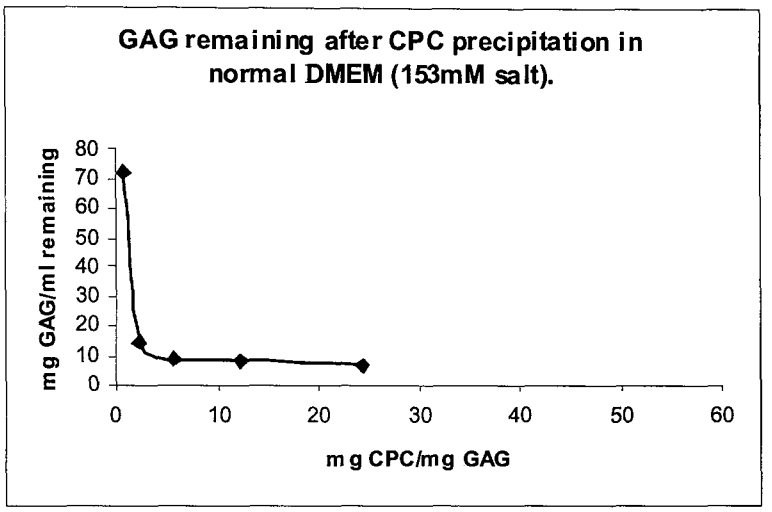
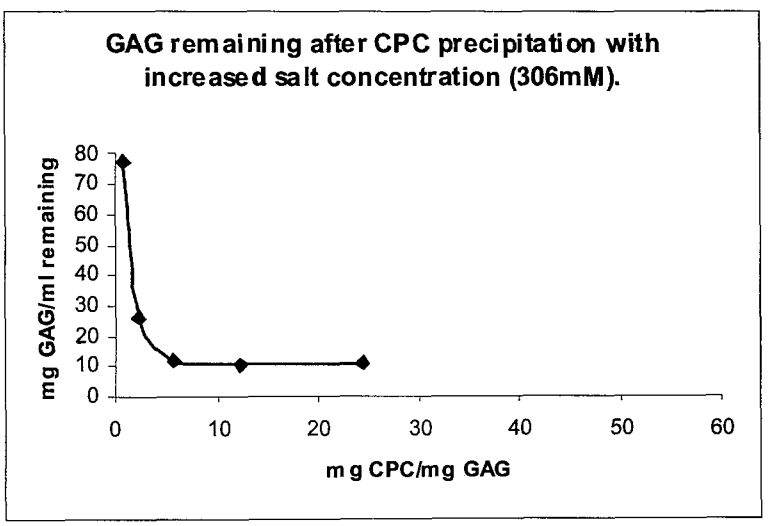


Fig 4.6. SDS-PAGE showing the effect of salt concentration upon CPC precipitation of proteins from explant medium in the presence of 158 mM salt (a) or 306 mM salt (b). Cartilage explants were treated with IL-1α (100 ng/ml) for 24 h. The medium was replaced with fresh medium and the cartilage cultured for a further 5 h. Explant medium was removed and its GAG content estimated. 400 µl explant medium/sample were precipitated at room temperature with 0-24.4 mg CPC/mg GAG. Two samples for each concentration were prepared. One sample was precipitated in normal explant medium (158 mM salt) (a) and the other in explant medium with double the salt concentration (306 mM) (b). The supernatant was removed and the proteoglycan rich pellet was treated with 0.4 M sodium acetate in 90 % ethanol to remove the complex bound CPC. Supernatants and pellets were analysed by SDS-PAGE on a 12.5 % gel. Gels were silver stained.



a

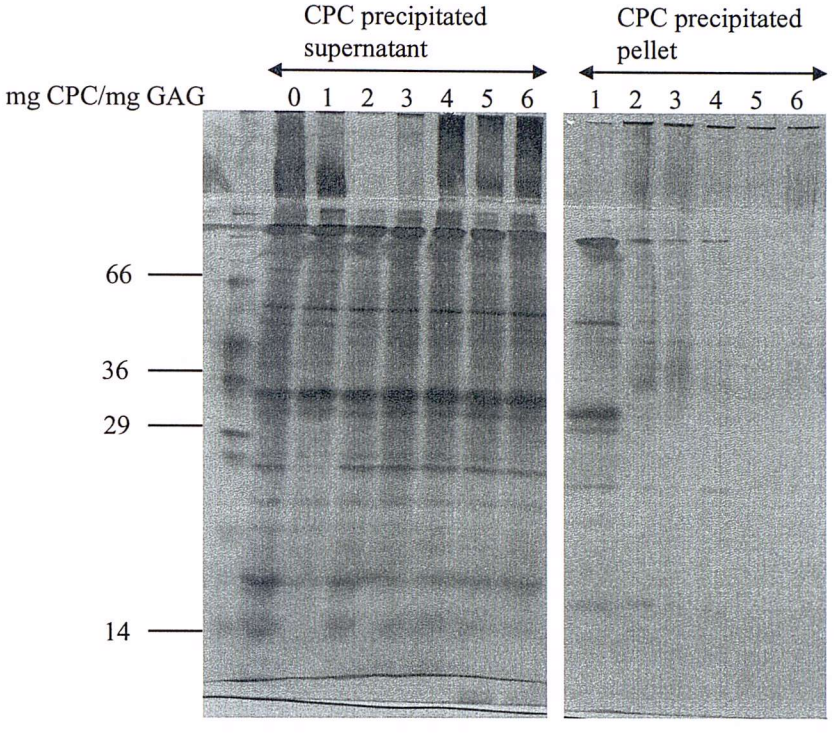


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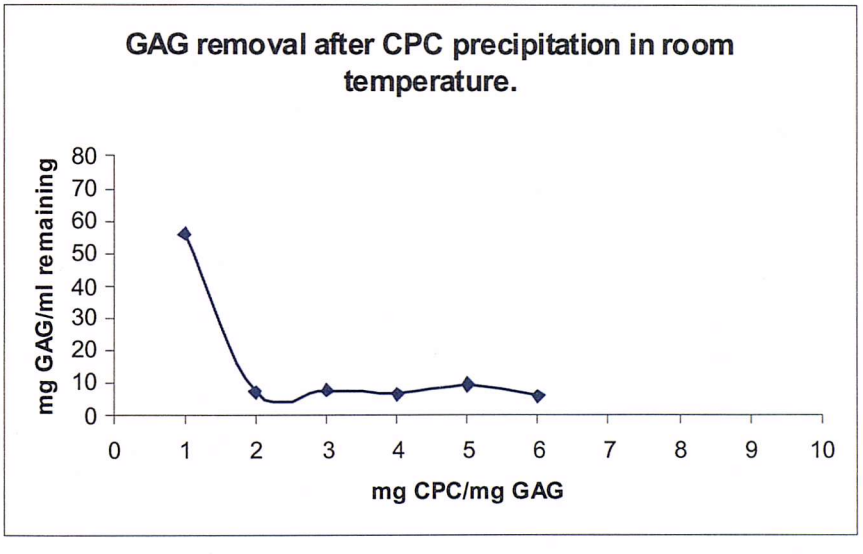
Fig 4.7. GAG remaining after CPC precipitation of explant medium at room temperature in normal explant medium (158 mM salt) (a) compared to explant medium with a two-fold higher salt concentration (306 mM) (b). After CPC precipitation with 0-24.4 mg CPC/mg GAG, the remaining GAG concentration was analysed with the DMB assay.

4.3.2 3 mg CPC/mg GAG chosen for optimal protein recovery and GAG removal

From the preceding experiments, it was clear that a CPC concentration between 2.4-5.7 mg CPC/mg GAG would be optimal for efficient removal of GAG together with good recovery of protein. To determine precisely what CPC concentration to use for future analysis, explant medium was prepared as before and treated with 1-6 mg CPC/mg GAG for 30 min at room temperature. (fig 4.8a). 1 mg CPC/mg GAG was too little to remove GAG efficiently (55 µg/ml left) (fig 4.8b) and the recovery of low molecular weight proteins was especially poor. In the pellet several proteins were detected with this concentration, but most disappeared when the CPC concentration was increased. With 2 mg CPC/mg GAG the GAG remaining in the supernatant was less than 10 µg/ml and no protein losses were detected. However, given the tendency of some proteins to co-precipitate at low CPC concentrations and the need to ensure efficient GAG removal, it was felt that 2 mg was borderline and therefore 3 mg CPC/mg GAG was chosen for future use.



a



b

Fig 4.8. SDS-PAGE showing the effect of CPC concentration upon precipitation of proteins from explant medium at room temperature (a) and the remaining GAG in the samples (b). Cartilage explants were treated with IL-1α (100 ng/ml) for 24 h. The medium was replaced with fresh medium and the cartilage cultured for a further 5 h. Explant medium was removed and its GAG content estimated with the DMB assay. 800 µl/sample were precipitated for 30 min at room temperature with 1-6 mg CPC/mg GAG. The supernatants were removed and the pellets were treated with 0.4 M sodium chloride in 90 % ethanol to remove the complex bound CP-ions. Supernatants and pellets were analysed by SDS-PAGE on a 12.5 % gel, and silver stained.

4.4 Effect of O_2 and medium supplements on protein biosynthetic patterns

At the time I was optimising the conditions for CPC precipitation, I also investigated the effect of the culture environment on the protein biosynthetic patterns. The need for culture medium supplements such as insulin and transferrin and the possible effects of reduced oxygen tension on gene expression were investigated. All previous experiments were done in a humidified atmosphere of 5 % CO_2 and 95 % air, but articular chondrocytes are adapted to a hypoxic environment (Silver, 1975) Furthermore, metabolic labelling requires serum-free medium.

Cartilage explants were cultured with or without insulin-transferrin-sodium selenite (ITS) supplement for 3 days, without change of medium. Samples were pulsed with [^{35}S]methionine/cysteine for 5 h, and the explant medium CPC precipitated and prepared for 2D-electrophoresis. This analysis was done before the CPC precipitation method was optimised, thus the protein patterns were generally weaker due to the relatively low protein concentration.

The control and ITS supplemented samples looked quite similar with silver staining (not shown) but ITS supplemented medium had stronger metabolic labelling, possibly indicating higher concentration of newly synthesised proteins (fig 4.9a and b). The ITS supplemented sample also displayed metabolic labelling of some proteins, especially those of low molecular mass that were absent from the control.

Cartilage explants were cultured anaerobically for 3 days (with ITS). The medium was changed after two days to mimic the standard explant stimulation procedure. Explants were washed with methionine and cysteine free medium for 30 min in normal atmosphere and then pulsed anaerobically for 5 hr with [^{35}S]methionine/cysteine. The supernatant was removed, CPC precipitated and prepared for 2D-electrophoresis. Anaerobically incubated explants gave a very similar protein pattern to control, possibly a little weaker (fig 4.10). The anaerobic condition did not seem to alter the protein pattern. All explant cultures from fig 4.4 and onwards were supplemented with ITS.

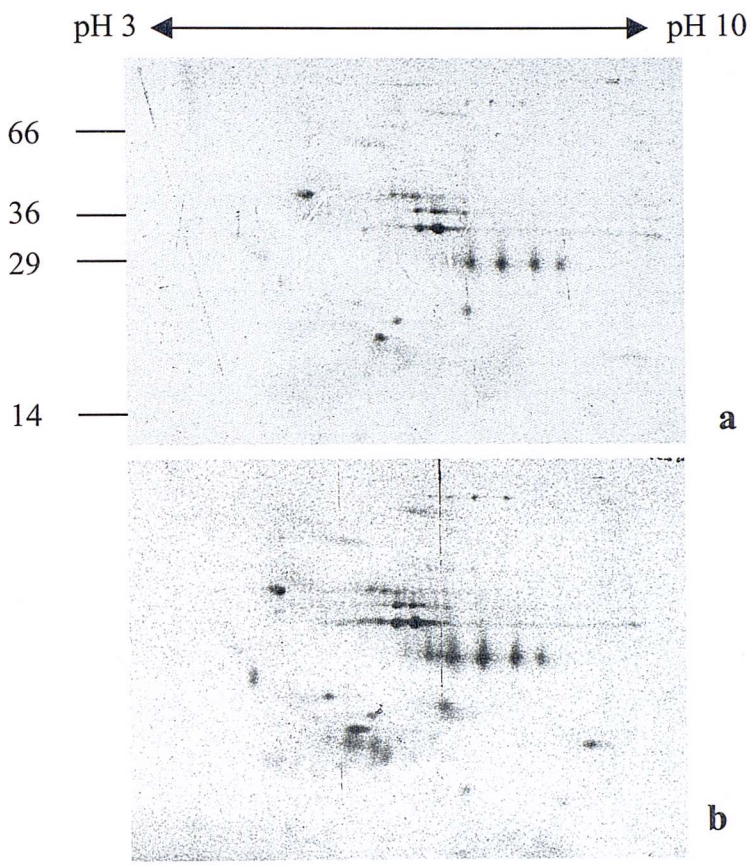


Fig 4.9. Autoradiographs of 2D-electrophoresis of explant medium cultured as usual (a), or with ITS medium supplement (b). 3 g of cartilage explant were cultured with or without ITS medium supplement for 3 days without change of medium. All cartilage explant samples were pulsed with [^{35}S]methionine/cysteine (100 µCi/ml) for 5 h as usual. Explant media was removed and CPC precipitated with 17-23 mg CPC/mg GAG. Excess CPC was precipitated on ice. Supernatants were dialysed against water and lyophilized. 2D-electrophoresis was performed using a pH 3-10 gradient in the first dimension and SDS-PAGE on a 12.5 % gel in the second. Gels were silver stained, dried and exposed to film for one week. Note that this analysis was performed prior to optimisation of the CPC precipitation method.

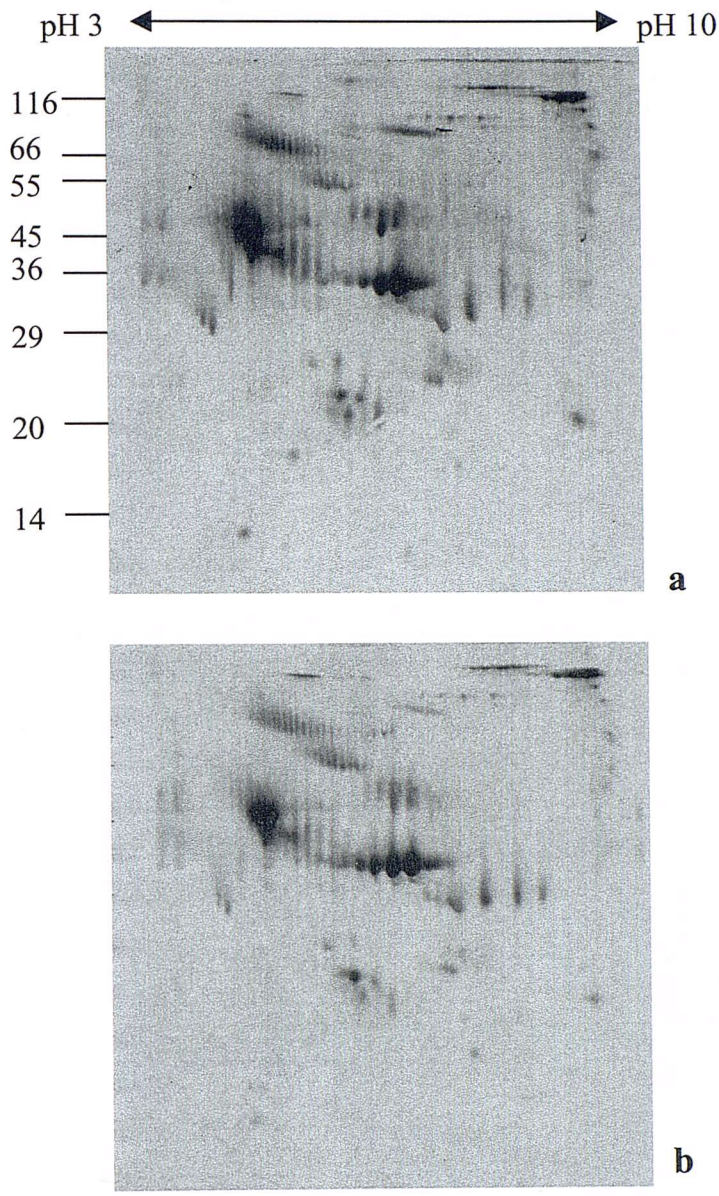


Fig 4.10. Autoradiographs of 2D-gels of cartilage explant medium cultured in the presence (a) or absence (b) of oxygen. 3 g of cartilage explant were cultured for 3 days in the presence or absence of oxygen and then pulsed with [35S]methionine/cysteine for 5 h under the same conditions. Explant medium was removed and CPC precipitated with 3 mg CPC/mg GAG. The supernatant was removed and dialysed against water overnight and lyophilized. 2D-electrophoresis was performed using a pH 3-10 gradient in the first dimension and SDS-PAGE on a 12.5 % gel in the second. Gels were stained with silver and exposed to film for 3 nights.

4.5 2D-electrophoresis of explant media after optimisation of CPC precipitation

Medium was prepared as before and treated with 3 mg CPC/mg GAG for 30 min at room temperature. The sample was then dialysed overnight at room temperature to removed detergent and salt (a previous experiment had shown that no degradation of proteins occurred when a sample was left at room temperature overnight, data not shown). After lyophilization, the sample was analysed by 2D-electrophoresis and the gel stained with silver. 100-200 protein spots were resolved, of which most were well focused (fig 4.11). The exception was a set of proteins in the middle of the gel that had not focused completely due to their relative overloading. These were later identified as the C-terminal propeptide of type II collagen: they are the major stainable and labelled protein spots of the porcine explant medium.

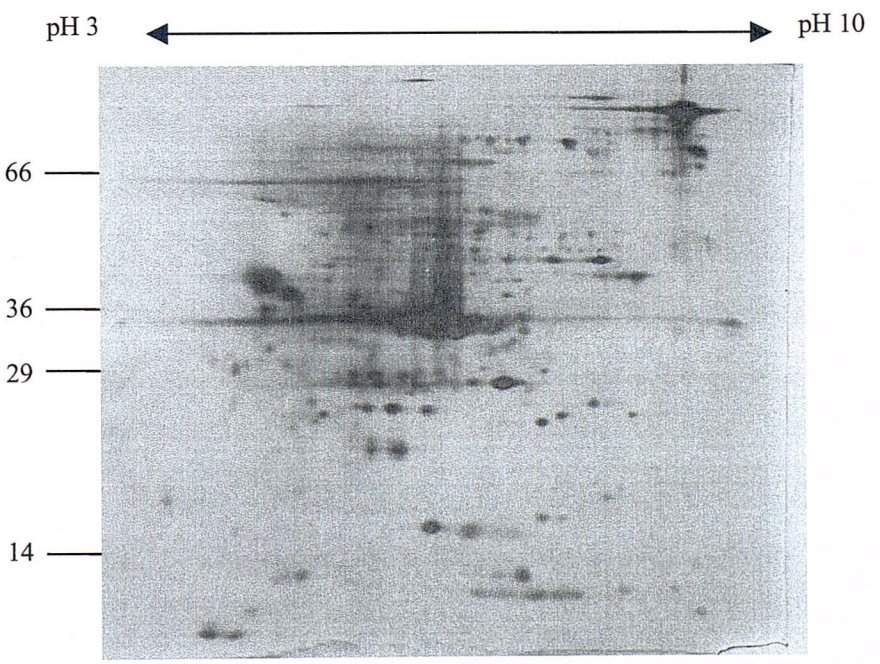


Fig 4.11. Silver stained 2D-gel of explant medium after CPC precipitation with 3 mg CPC/mg GAG. 4 g of porcine articular cartilage explants were treated with IL-1α (100 ng/ml) for 24 h. The medium was replaced with fresh medium and the cartilage cultured for a further 5 h. Explant medium was removed and its GAG content estimated. The medium sample was CPC precipitated with 3 mg CPC/mg GAG (corresponding to 2.1 mg CPC) for 30 min at room temperature. The supernatant was removed and dialysed against water at room temperature and lyophilized. 65 µg of protein was separated on 3-10 pH gradient and focused proteins were electrophoresed by SDS-PAGE on a 12.5 % gel.

Having established optimal conditions for removing GAG with minimal loss of proteins, the experiment was repeated with metabolically labelled explant medium samples treated with or without IL-1 (fig 4.12). Repeated experiments showed that the silver stained protein pattern on 2D was very reproducible (fig 4.12 a and b versus fig 4.11). Proteins detected with silver staining but not by autoradiography were not synthesised by the chondrocytes during the 5 h pulse (fig 4.12 a-d). This could have been due to a very low rate of synthesis or they may have been non-chondrocyte proteins (e.g. plasma proteins, synovial proteins) present in the cartilage matrix. Spots detected by autoradiography but not by silver stain probably contained less than 100 fmol of protein.

Only one labelled IL-1 regulated protein of about 55 kDa was seen (fig 4.12b and d). This protein was identified as MMP-1 by MALDI and ESI MS. MMP-1 was previously identified from a 1D-gel (*chapter 3*). Surprisingly, one of the major secreted proteins (SAA), which by 1D-electrophoresis showed to be regulated by IL-1 (*chapter 3*), was not detected on the 2D-gels. 1D-electrophoresis of the material had revealed that it was equally distributed between the supernatant and pellet after CPC precipitation (data not shown). Although this lowered the chances of detection on 2D-gels, Ducret et al '96 successfully studied SAA isoforms in serum by 2D-electrophoresis. So the proteins should isoelectric focus. Gp-38, in contrast to its abundance on the 1D-gels, was detected as a relatively faint labelled spot at the basic end of the 2D-gel. This IL-1 regulated protein is very basic (calculated pI=9.17) and was not always seen on a pH 3-10 gradient. It should be detected more efficiently on a more basic gradient. All major silver stained protein spots from the gels in fig 4.12 were excised and analysed by mass spectrometry.

Of the proteins identified, collagen type II, C-terminal propeptide of collagen type II, osteonectin/SPARC, TIMP-1, CTGF and MMP-1 were metabolically labelled (fig 4.12d). Most of the stained proteins were not labelled.

MALDI and ESI MS/MS identified 103 different proteins or protein subunits from the IL-1 treated cartilage explant media sample (fig 4.13, table 4.1). Full details of protein identifications are listed in Appendix 1. Some unlabelled matrix proteins were identified including clusterin, fibromodulin, COMP and thrombospondin-1.

However it was not possible to know if they were derived from chondrocytes or from synovial fluid diffusing into the cartilage. They may not be easily removed by washing of the cartilage explants. This was especially reflected by the abundance of haemoglobin. Several medium changes before the metabolic labelling failed to wash away haemoglobin from the explants.

Many enzymes involved in the glycolytic pathway such as triose phosphate isomerase, phosphoglycerate kinase and enolase (alpha and beta) were identified. These cytosolic proteins suggested that there could be ongoing cell death.

The large number of proteins detected on the gel that were possibly not cartilage derived hindered identification of more cartilage specific proteins. Any attempt to increase the amount of protein loaded on to the gel, would increase the concentration of all proteins detected, not just those secreted by the chondrocytes.

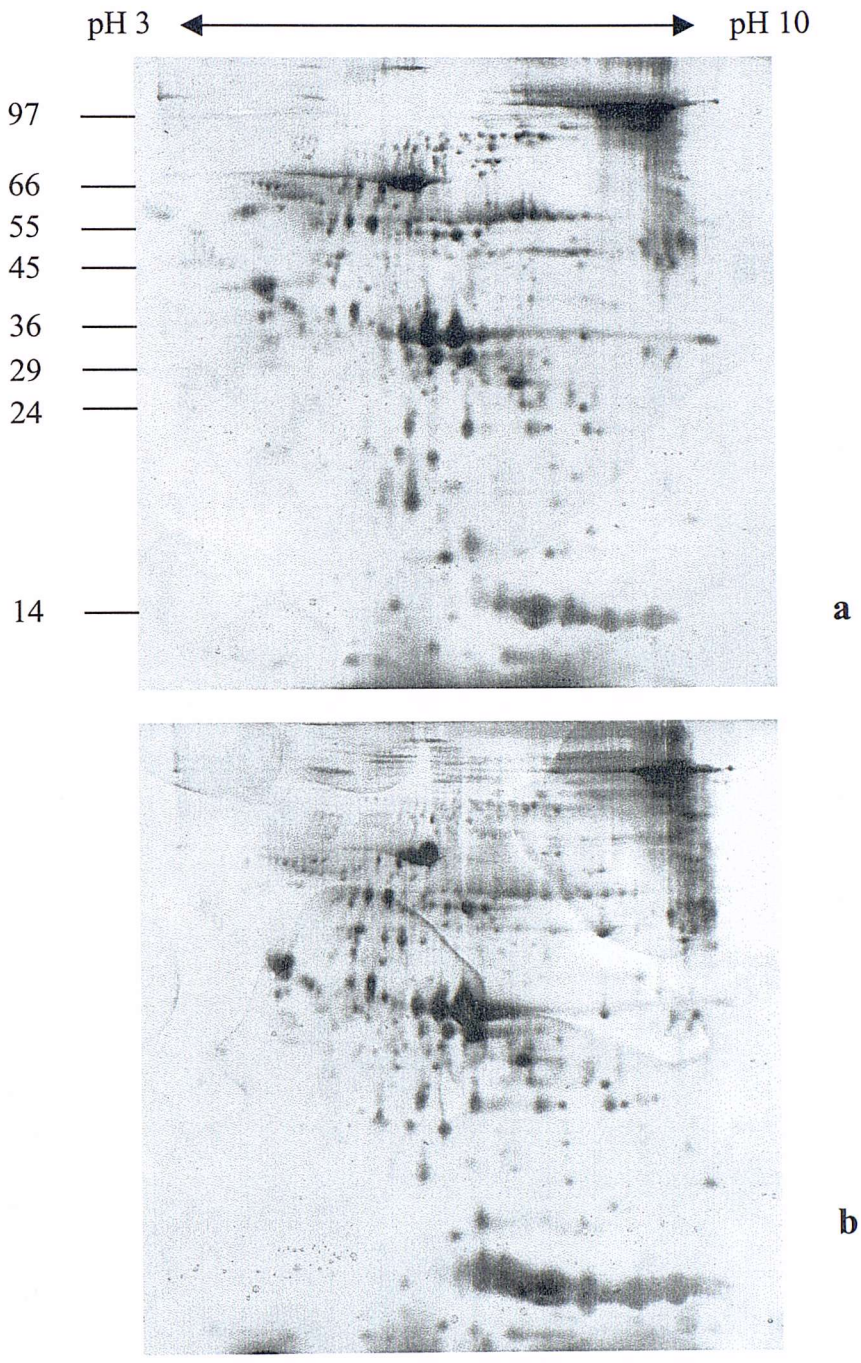
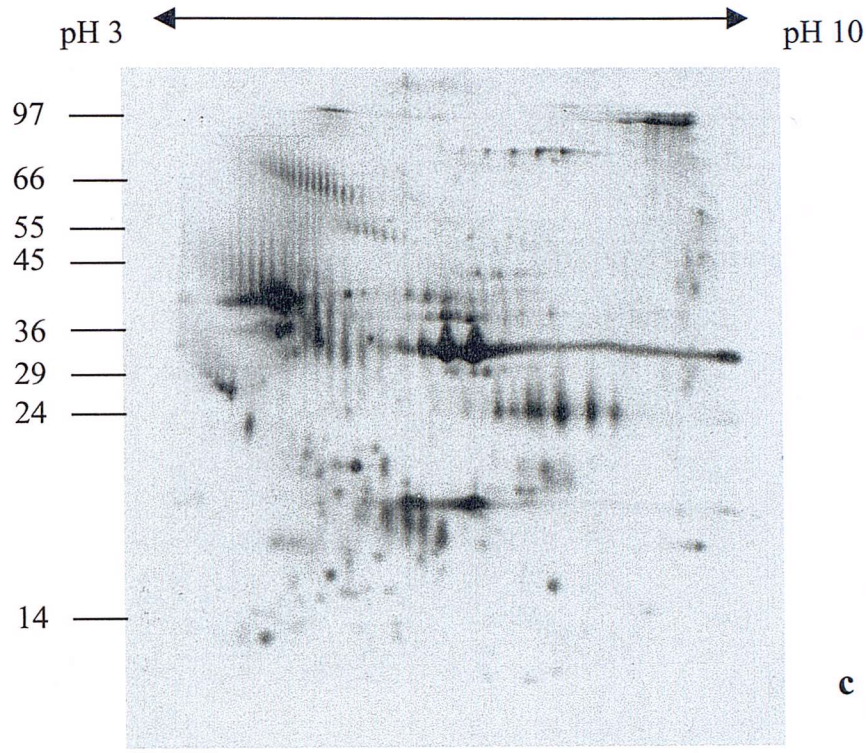


Fig 4.12. Silver stained 2D-gel of proteins secreted from control (a) and IL-1 treated (b) explant media. Their corresponding autoradiographs are shown in (c) and (d). 6 g of porcine articular cartilage were cultured with or without IL-1 overnight and then pulsed with [^{35}S]methionine/cysteine (200 µCi/ml) for 5 h. Explant medium was removed and treated with 3mg CPC/mg GAG for 30 min at room temperature. The supernatant was removed and dialysed against water overnight at 4°C and lyophilized. 60 µg of protein was used for isoelectric focusing on a pH 3-10 gradient. Focused proteins were electrophoresed by SDS-PAGE on a 12.5 % gel. The gel was stained with silver, dried and exposed to film for 1 week. The gel was rehydrated and protein spots excised. Proteins were identified by MALDI and ESI MS.



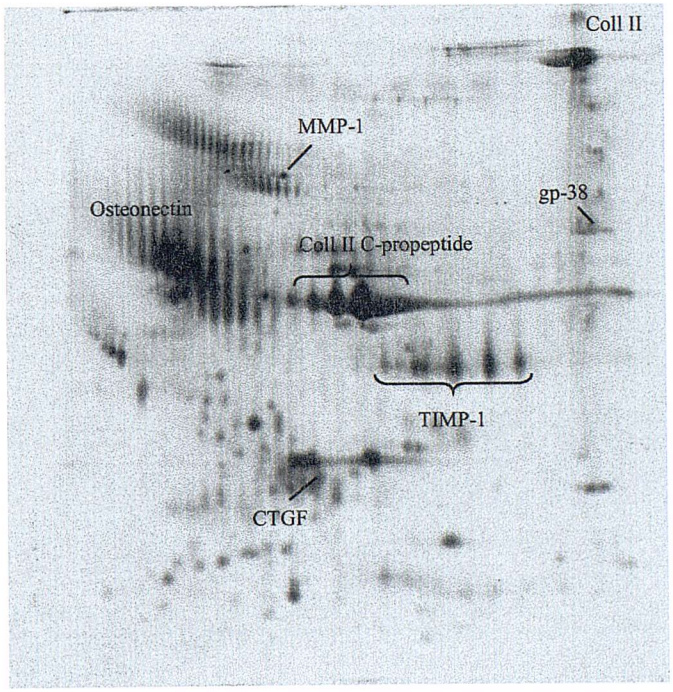


Fig 4.12 continued. Silver stained 2D-gel of proteins secreted from control (a) and IL-1 treated (b) explant medium. Their corresponding autoradiographs are shown in (c) and (d).

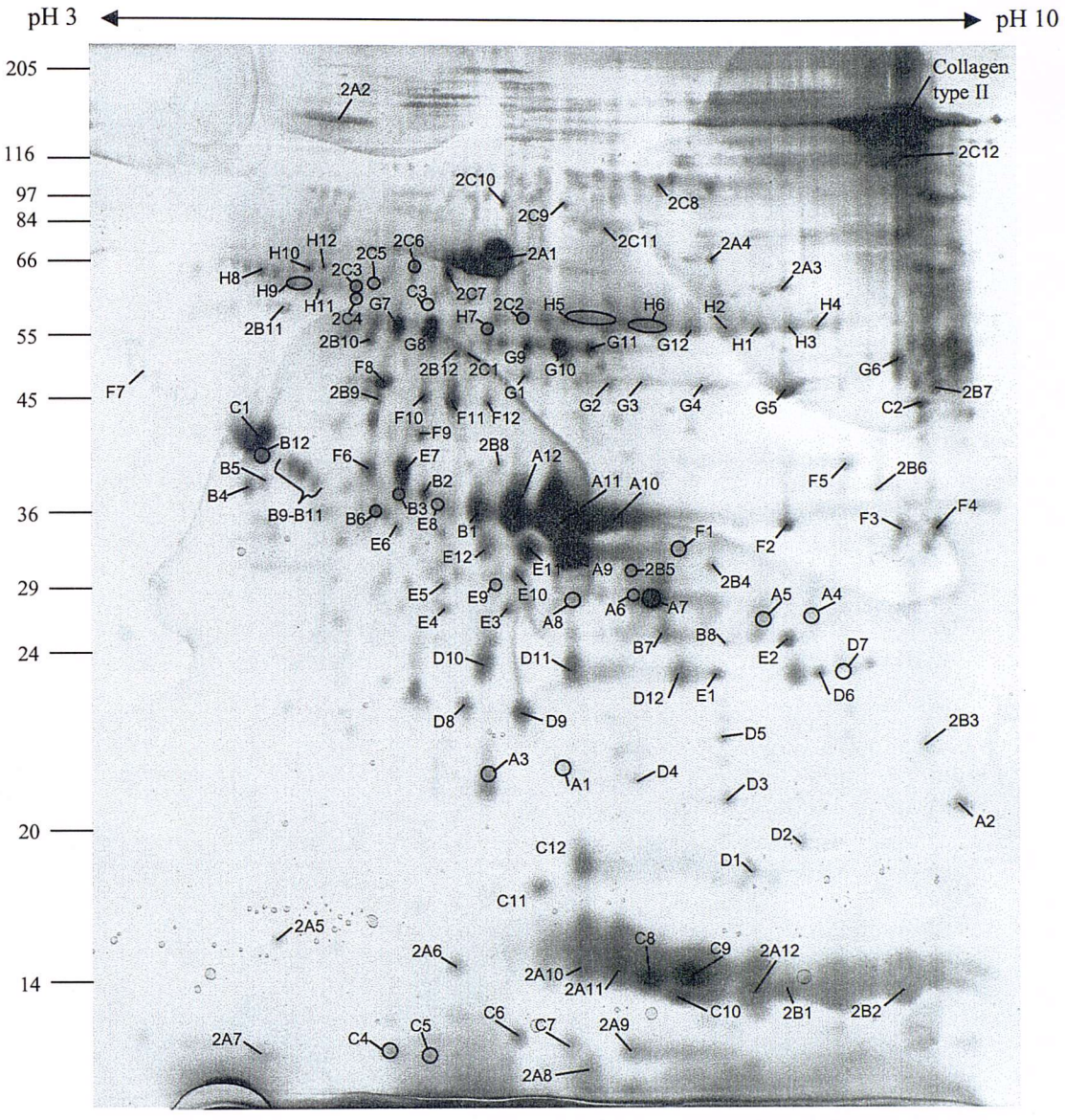


Fig 4.13. Silver stained 2D-gel of proteins secreted from IL-1 treated explant medium (enlargement of fig 4.12b). The preparation of the sample is described in legend of fig 4.12. Proteins were identified by MALDI and ESI MS/MS. Identified proteins corresponding to the numbers on the gel are listed in table 4.1.

Table 4.1 Proteins identified from explant medium by MALDI and ESI MS/MS.
a. by spot number. b. in alphabetical order
Table 4.1a

Spot	Protein
A1	Connective tissue growth factor (CTGF)
A2	Peptidyl-prolyl cis-trans isomerase B Cartilage oligomeric matrix protein (COMP)
A3	Connective tissue growth factor (CTGF) Tetranectin
A4	Metalloproteinase inhibitor 1 (TIMP-1)
A5	Metalloproteinase inhibitor 1 (TIMP-1) Immunoglobulin
A6	Triosephosphate isomerase Collagen alpha 1 type II C-propeptide Haemoglobin beta chain
A7	Triosephosphate isomerase
A8	Phosphoglycerate mutase Triosephosphate isomerase
A9	Collagen alpha 1 type II C-propeptide
A10	Collagen alpha 1 type II C-propeptide
A11	Collagen alpha 1 type II C-propeptide
A12	Collagen alpha 1 type II C-propeptide Malate dehydrogenase Serum albumin
B1	Collagen alpha 1 type II C-propeptide
B2	L-lactate dehydrogenase B chain Collagen alpha 1 type II C-propeptide
B3	Collagen alpha 1 type II C-propeptide Thrombospondin 1 Collagen alpha 1 (XI) chain
B4	Collagen alpha 1 (XI) chain
B5	Collagen alpha 1 (XI) chain
B6	Annexin A8 Thrombospondin 1 Collagen alpha 1 (XI) chain
B7	Collagen alpha 1 (XI) chain
B8	Angiopoietin-related protein 2 Glutathione S-transferase P Collagen alpha 1 (XI) chain
B9	Osteonectin/SPARC Clusterin Heat shock 70 kDa protein
B10	Osteonectin/SPARC
B11	Osteonectin/SPARC
B12	Osteonectin/SPARC
C1	OsteonectinSPARC
C2	Aspartate aminotransferase
C3	Matrix metalloproteinase-1 (MMP-1)
C4	Polyubiquitin
C5	Polyubiquitin
C6	Calpactin 1 light chain
C7	Elongation factor 1-delta
C8	Haemoglobin beta chain
C9	Haemoglobin beta chain Haemoglobin epsilon chain
C10	Haemoglobin beta chain
C11	Superoxide dismutase [Cu-Zn] Haemoglobin beta chain
C12	Haemoglobin beta chain
D1	Fibrogen A-alpha-chain Nucloeside diphosphate kinase B
D2	Cofilin, non-muscle isoform
D3	Phosphatidylethanolamine-binding protein
D4	Peroxiredoxin 1 Peroxiredoxin 4
D5	Peroxiredoxin 1 Peroxiredoxin 4
D6	Peroxiredoxin 1 Peroxiredoxin 4
D7	Peroxiredoxin 1 Ig lambda chain C region Peroxiredoxin 4
D8	DJ-1 protein
D9	RNA-binding protein regulatory subunit
D10	Ig lambda chain C region Angiopoietin 1 receptor Thioredoxin-dependent peroxide reductase
D11	Ig lambda chain C region
D12	Ig lambda chain C region
E1	Superoxide dismutase [Mn], mitochondrial
E2	Glutathione S-transferase P
E3	Thrombospondin 1
E4	Thrombospondin 1
E5	Thrombospondin 1
E6	Thrombospondin 1
E7	Thrombospondin-1 Inorganic pyrophosphatase
E8	Collagen alpha 1 type II C-propeptide F-actin capping protein alpha-2 subunit Thrombospondin 1
E9	Phosphoglycerate mutase Non-selenium glutathione phospholipid hydroxyperoxide peroxidase
E10	Phosphoglycerate mutase
E11	Collagen alpha 1 type II C-propeptide
E12	Collagen alpha 1 type II C-propeptide
F1	Collagen alpha 1 type II C-propeptide Type IX collagen alpha 1 chain
F2	L-lactate dehydrogenase A chain
F3	60S acidic ribosomal protein P0
F4	Chondroadherin
F5	Fructose-bisphosphate aldolase A
F6	Procollagen C-proteinase enhancer protein Annexin A8
F7	Alpha-1 acid glycoprotein
F8	Actin beta chain
F9	Alpha enolase
F10	Fibrinogen beta chain
F11	Fibrinogen beta chain
F12	Fibrinogen beta chain
G1	Ornithine aminotransferase, mitochondrial
G2	NADP+-dependent isocitrate dehydrogenase Fumarylacetoacetase Phosphoglycerate kinase 1
G3	Isocitrate dehydrogenase Phosphoglycerate kinase 1
G4	Phosphoglycerate kinase 1
G5	Phosphoglycerate kinase 1
G6	47 kDa heat shock protein
G7	Similar to glucose regulated protein, 58 kDa Alpha-1-antitrypsin
G8	Similar to glucose regulated protein, 58 kDa Alpha-1-antitrypsin
G9	Alpha enolase Beta enolase Phosphopyruvate hydratase

G10	Alpha enolase Beta enolase Phosphopyruvate hydratase
G11	Alpha enolase Beta enolase Phosphopyruvate hydratase
G12	Ig heavy chain V region Alpha enolase
H1	Ig heavy chain V region Protein disulphide isomerase A3
H2	Ig heavy chain V region
H3	Ig heavy chain V region
H4	Ig heavy chain V region
H5	Procollagen C-proteinase enhancer protein P Beta-2-glycoprotein 1 Ig heavy chain V region
H6	Ig heavy chain V region Procollagen C-proteinase enhancer protein P
H7	Serum albumin Procollagen C-proteinase enhancer protein P Ig heavy chain V region Aldehyde dehydrogenase, mitochondrial
H8	Alpha-2-HS-glycoprotein
H9	Alpha-1-antichymotrypsin 3
H10	Alpha-1-antichymotrypsin 3 Nucleobindin 1 (CALNUC)
H11	Alpha-1-antichymotrypsin 3 78 kDa glucose-regulated protein Fibromodulin
H12	Fibromodulin RAB GDP dissociation inhibitor alpha 78 kDa glucose-regulated protein Alpha-1-antichymotrypsin 3
2A1	Serum albumin
2A2	Serum albumin Collagen alpha 1 (VI) chain
2A3	Pyruvate kinase, M2 isozyme Serum albumin
2A4	Serum albumin
2A5	Vimentin
2A6	Cystatin B Haemoglobin beta chain
2A7	Cartilage oligomeric matrix protein (COMP)
2A8	Haemoglobin beta chain
2A9	Polyubiquitin

2A10	Haemoglobin beta chain
2A11	Haemoglobin beta chain
2A12	Haemoglobin alpha chain Haemoglobin beta chain
2B1	Haemoglobin alpha chain
2B2	Haemoglobin alpha chain
2B3	Haemoglobin beta chain
2B4	Dihydropteridine reductase
2B5	Collagen alpha 1 type II C-propeptide Adenylate kinase isoenzyme 2, mitochondrial
2B6	Glyceraldehyde 3-phosphate dehydrogenase Ornithine decarboxylase
2B7	38 kDa heparin-binding glycoprotein (Gp38)
2B8	Transaldolase Serum albumin
2B9	Actin, cytoplasmic 2
2B10	Gelsolin, plasma Glutathione synthetase Protein disulphide isomerase A6
2B11	Protein disulphide isomerase
2B12	Pigment epithelium-derived factor Transthyretin (prealbumin) Alpha enolase
2C1	RAB GDP dissociation inhibitor alpha Alpha enolase
2C2	Cytosol aminopeptidase Leucine aminopeptidase
2C3	Matrix metalloproteinase-3 (MMP-3) Alpha-1-antichymotrypsin 3
2C4	Vitamin D-binding protein Alpha-1-antitrypsin
2C5	Antithrombin-III 60 kDa heat shock protein
	Lysyl oxidase homolog
2C6	Lysyl oxidase homolog 2
2C7	Serum albumin Prolyl 4-hydroxylase alpha subunit
2C8	Collagen alpha 2 (XI) chain
2C9	Serotransferrin
2C10	Serum albumin
2C11	Serotransferrin Inhibitor of carbonic anhydrase Lactotransferrin
2C12	Type IX collagen alpha 1 chain

Table 4.1b

Spot	Protein
2B7	38 kDa heparin-binding glycoprotein (Gp38)
G6	47 kDa heat shock protein
2C5	60 kDa heat shock protein
F3	60S acidic ribosomal protein P0
H11	78 kDa glucose-regulated protein
H12	78 kDa glucose-regulated protein
F8	Actin beta chain
2B9	Actin, cytoplasmic 2
2B5	Adenylate kinase isoenzyme 2, mitochondrial
H7	Aldehyde dehydrogenase, mitochondrial
F9	Alpha enolase
G9	Alpha enolase
G10	Alpha enolase
G11	Alpha enolase
G12	Alpha enolase
2B12	Alpha enolase
2C1	Alpha enolase
F7	Alpha-1 acid glycoprotein
H9	Alpha-1-antichymotrypsin 3
H10	Alpha-1-antichymotrypsin 3
H11	Alpha-1-antichymotrypsin 3
H12	Alpha-1-antichymotrypsin 3
2C3	Alpha-1-antichymotrypsin 3
G7	Alpha-1-antitrypsin
G8	Alpha-1-antitrypsin
2C4	Alpha-1-antitrypsin
H8	Alpha-2-HS-glycoprotein
D10	Angiopoietin 1 receptor
B8	Angiopoietin-related protein 2
B6	Annexin A8
F6	Annexin A8
2C5	Antithrombin-III
C2	Aspartate aminotransferase
G9	Beta enolase
G10	Beta enolase
G11	Beta enolase
H5	Beta-2-glycoprotein 1
C6	Calpactin 1 light chain
A2	Cartilage oligomeric matrix protein (COMP)
2A7	Cartilage oligomeric matrix protein (COMP)
F4	Chondroadherin
B9	Clusterin
D2	Cofilin, non-muscle isoform
2A2	Collagen alpha 1 (VI) chain
B3	Collagen alpha 1 (XI) chain
B4	Collagen alpha 1 (XI) chain
B5	Collagen alpha 1 (XI) chain
B6	Collagen alpha 1 (XI) chain
B7	Collagen alpha 1 (XI) chain
B8	Collagen alpha 1 (XI) chain
A6	Collagen alpha 1 type II C-propeptide
A9	Collagen alpha 1 type II C-propeptide
A10	Collagen alpha 1 type II C-propeptide
A11	Collagen alpha 1 type II C-propeptide
A12	Collagen alpha 1 type II C-propeptide
B1	Collagen alpha 1 type II C-propeptide
B2	Collagen alpha 1 type II C-propeptide
B3	Collagen alpha 1 type II C-propeptide
E8	Collagen alpha 1 type II C-propeptide
E11	Collagen alpha 1 type II C-propeptide
E12	Collagen alpha 1 type II C-propeptide
F1	Collagen alpha 1 type II C-propeptide
2B5	Collagen alpha 1 type II C-propeptide
2C8	Collagen alpha 2 (XI) chain
A1	Connective tissue growth factor (CTGF)
A3	Connective tissue growth factor (CTGF)
2A6	Cystatin B
2C2	Cytosol aminopeptidase
2B4	Dihydropteridine reductase
D8	DJ-1 protein
C7	Elongation factor 1-delta
E8	F-actin capping protein alpha-2 subunit
F10	Fibrinogen beta chain
F11	Fibrinogen beta chain
F12	Fibrinogen beta chain
D1	Fibrogen A-alpha-chain
H11	Fibromodulin
H12	Fibromodulin
F5	Fructose-bisphosphate aldolase A
G2	Fumarylacetoacetase
2B10	Gelsolin, plasma
B8	Glutathione S-transferase P
E2	Glutathione S-transferase P
2B10	Glutathione synthetase
2B6	Glyceraldehyde 3-phosphate dehydrogenase
2A12	Haemoglobin alpha chain
2B1	Haemoglobin alpha chain
2B2	Haemoglobin alpha chain
A6	Haemoglobin beta chain
C8	Haemoglobin beta chain
C9	Haemoglobin beta chain
C10	Haemoglobin beta chain
C11	Haemoglobin beta chain
C12	Haemoglobin beta chain
2A6	Haemoglobin beta chain
2A8	Haemoglobin beta chain
2A10	Haemoglobin beta chain
2A11	Haemoglobin beta chain
2A12	Haemoglobin beta chain
2B3	Haemoglobin beta chain
C9	Haemoglobin epsilon chain
B9	Heat shock 70 kDa protein
G12	Ig heavy chain V region
H1	Ig heavy chain V region
H2	Ig heavy chain V region
H3	Ig heavy chain V region
H4	Ig heavy chain V region
H5	Ig heavy chain V region
H6	Ig heavy chain V region
H7	Ig heavy chain V region
D7	Ig lambda chain C region
D10	Ig lambda chain C region
D11	Ig lambda chain C region
D12	Ig lambda chain C region
A5	Immunoglobulin
2C11	Inhibitor of carbonic anhydrase
E7	Inorganic pyrophosphatase
G3	Isocitrate dehydrogenase
2C11	Lactotransferrin
2C2	Leucine aminopeptidase
F2	L-lactate dehydrogenase A chain
B2	L-lactate dehydrogenase B chain
2C5	Lysyl oxidase homolog 2

2C6	Lysyl oxidase homolog 2
A12	Malate dehydrogenase
C3	Matrix metalloproteinase-1 (MMP-1)
2C3	Matrix metalloproteinase-3 (MMP-3)
A4	Metalloproteinase inhibitor 1 (TIMP-1)
A5	Metalloproteinase inhibitor 1 (TIMP-1)
G2	NADP+-dependent isocitrate dehydrogenase
E9	Non-selenium glutathione phospholipid hydroxyperoxide peroxidase
H10	Nucleobindin 1 (CALNUC)
D1	Nucloeside diphosphate kinase B
G1	Ornithine aminotransferase, mitochondrial
2B6	Ornithine decarboxylase
B9	Osteonectin/SPARC
B10	Osteonectin/SPARC
B11	Osteonectin/SPARC
B12	Osteonectin/SPARC
C1	OsteonectinSPARC
A2	Peptidyl-prolyl cis-trans isomerase B
D4	Peroxiredoxin 1
D5	Peroxiredoxin 1
D6	Peroxiredoxin 1
D7	Peroxiredoxin 1
D4	Peroxiredoxin 4
D5	Peroxiredoxin 4
D6	Peroxiredoxin 4
D7	Peroxiredoxin 4
D3	Phosphatidylethanolamine-binding protein
G2	Phosphoglycerate kinase 1
G3	Phosphoglycerate kinase 1
G4	Phosphoglycerate kinase 1
G5	Phosphoglycerate kinase 1
A8	Phosphoglycerate mutase
E9	Phosphoglycerate mutase
E10	Phosphoglycerate mutase
G9	Phosphopyruvate hydratase
G10	Phosphopyruvate hydratase
G11	Phosphopyruvate hydratase
2B12	Pigment epithelium-derived factor
C4	Polyubiquitin
C5	Polyubiquitin
2A9	Polyubiquitin
F6	Procollagen C-proteinase enhancer protein
H5	Procollagen C-proteinase enhancer protein P
H6	Procollagen C-proteinase enhancer protein P
H7	Procollagen C-proteinase enhancer protein P
2C7	Prolyl 4-hydroxylase alpha subunit
2B11	Protein disulphide isomerase
H1	Protein disulphide isomerase A3
2B10	Protein disulphide isomerase A6
2A3	Pyruvate kinase, M2 isozyme
H12	RAB GDP dissociation inhibitor alpha
2C1	RAB GDP dissociation inhibitor alpha
D9	RNA-binding protein regulatory subunit
2C9	Serotransferrin
2C11	Serotransferrin
A12	Serum albumin
H7	Serum albumin
2A1	Serum albumin
2A2	Serum albumin
2A3	Serum albumin
2A4	Serum albumin
2B8	Serum albumin
2C7	Serum albumin
2C10	Serum albumin
G7	Similar to glucose regulated protein, 58 kDa
G8	Similar to glucose regulated protein, 58 kDa
C11	Superoxide dismutase [Cu-Zn]
E1	Superoxide dismutase [Mn], mitochondrial
A3	Tetranectin
D10	Thioredoxin-dependent peroxide reductase
B3	Thrombospondin 1
B6	Thrombospondin 1
E3	Thrombospondin 1
E4	Thrombospondin 1
E5	Thrombospondin 1
E6	Thrombospondin 1
E8	Thrombospondin 1
E7	Thrombospondin-1
2B8	Transaldolase
2B12	Transthyretin (prealbumin)
A6	Triosephosphate isomerase
A7	Triosephosphate isomerase
A8	Triosephosphate isomerase
F1	Type IX collagen alpha 1 chain
2C12	Type IX collagen alpha 1 chain
2A5	Vimentin
2C4	Vitamin D-binding protein

4.6 2D-electrophoresis of explant media using pH 6-11 and pH 4-7 gradients

Proteins were run on a pH 6-11 gradient in order to detect gp-38 and other more basic proteins (fig 4.14a-d). The basic end of the 2D-gel focused very poorly. Gp-38 was detected as two protein spots. They were modestly upregulated in the IL-1 treated cartilage explant media sample compared to the control sample (fig 4.14 b and d). No candidate for SAA was found. Other labelled proteins identified included collagen type II, C-propeptide of collagen type II, and TIMP-1.

2D-electrophoresis using the pH 4-7 gradient produced well-focused gels (fig 4.15). Using a narrower gradient over the same distance meant that the protein spots of the C-propeptide of type II collagen were better separated. This allowed improved analysis of protein spots in the vicinity of the C-propeptide spots, which otherwise usually co-migrated on a pH 3-10 gradient. However, no additional IL-regulated protein spots were found using the pH 4-7 gradient. Identified labelled proteins included C-propeptide of collagen type II and osteonectin. A likely candidate for MMP-1 was present (fig 4.15d) but the corresponding silver-stained spot was not identified.

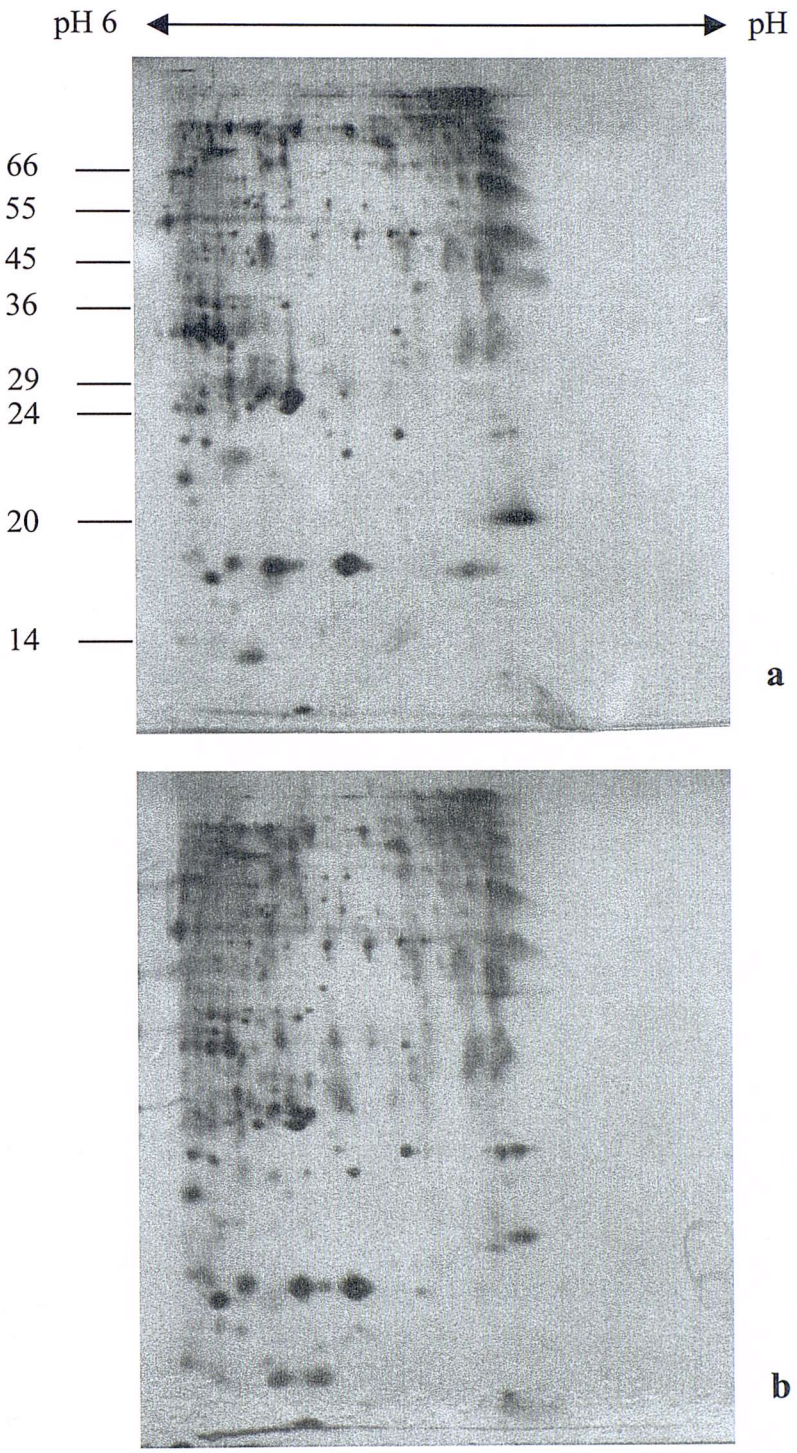


Fig 4.14. Silver stained 2D-gels (pH 6-11 gradient) of proteins from control (a) and IL-1 treated explant media (b). Their corresponding autoradiographs are shown in (c) and (d) respectively. 3 g of porcine articular cartilage were treated without (a and c) or with IL-1α (b and d) for 20 h and the pulsed with [^{35}S]methionine/cysteine (200 μCi/ml) for 5 h. Explant medium was removed and treated with 3 mg CPC/mg GAG for 30 min at room temperature. The supernatant was removed and dialysed against water at room temperature and lyophilized. Protein samples were isoelectrically focused on a pH 6-11 gradient. Focused proteins were electrophoresed by SDS-PAGE on a 12.5 % gel. The gels were stained with silver, dried and exposed to film for 1 week. Proteins identified by ESI MS are labelled.

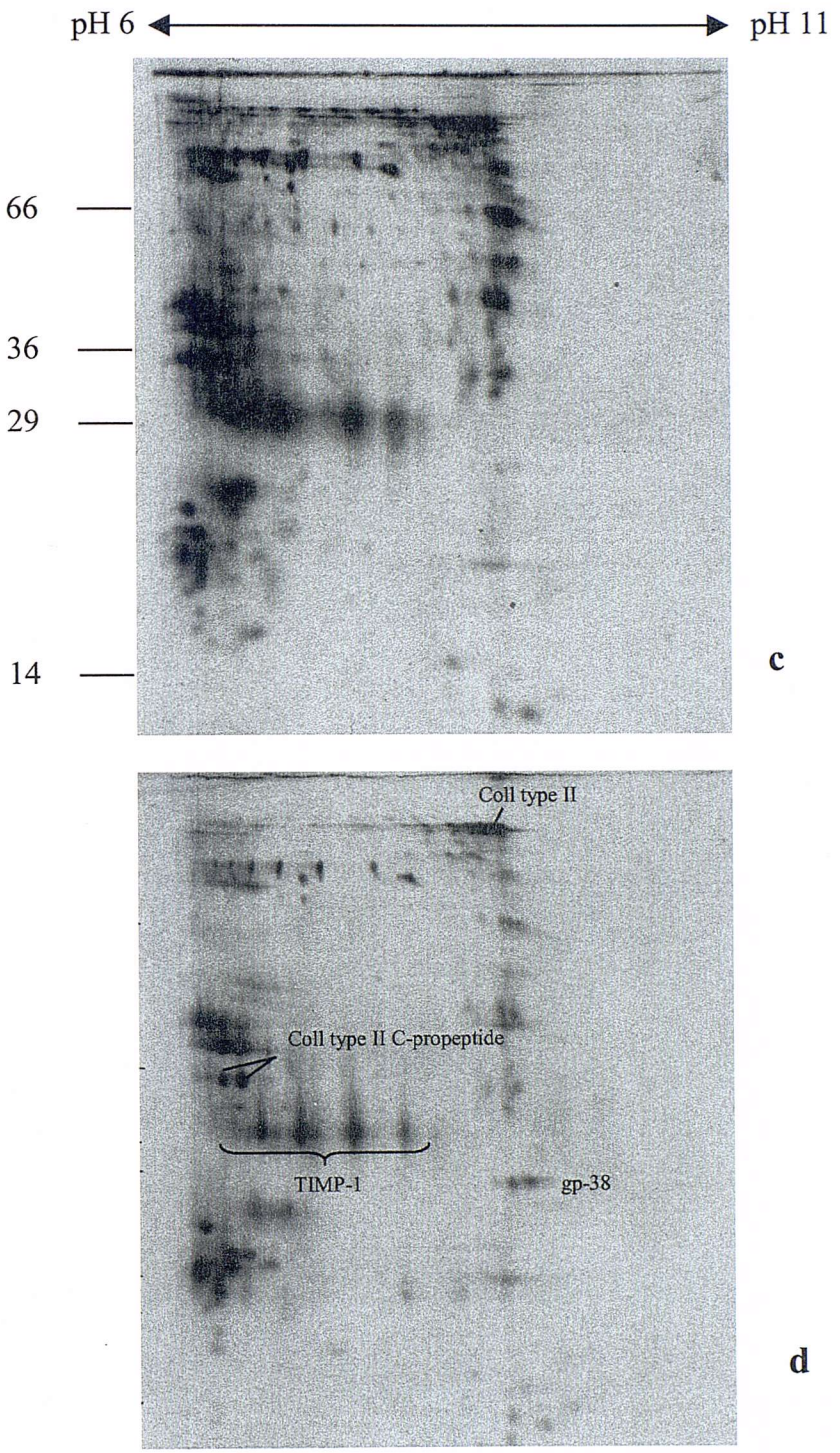


Fig 4.14 continued. Silver stained 2D-gels of proteins from control (a) and IL-1 treated explant medium (b). Their corresponding autoradiographs are shown in (c) and (d) respectively.

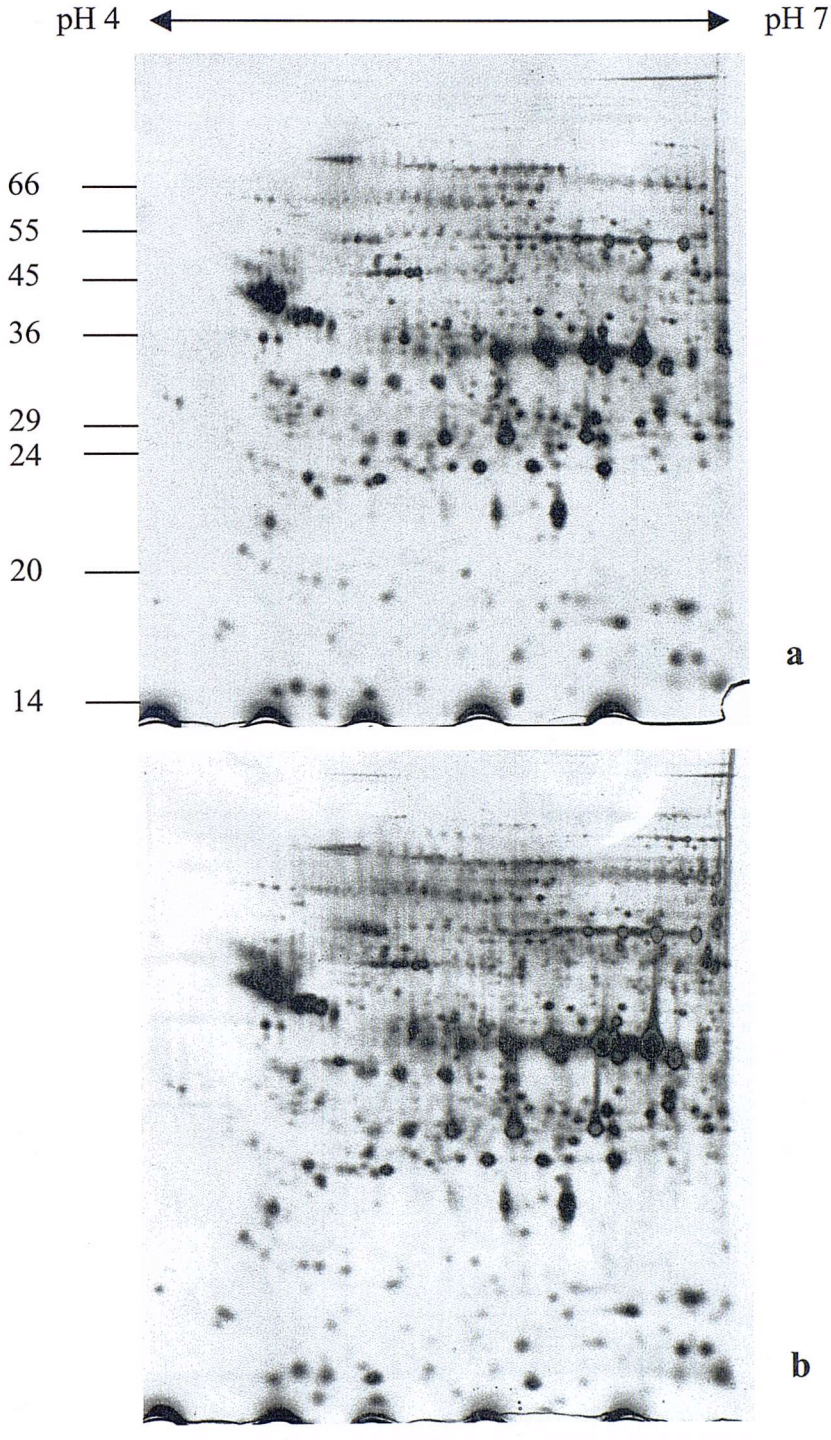
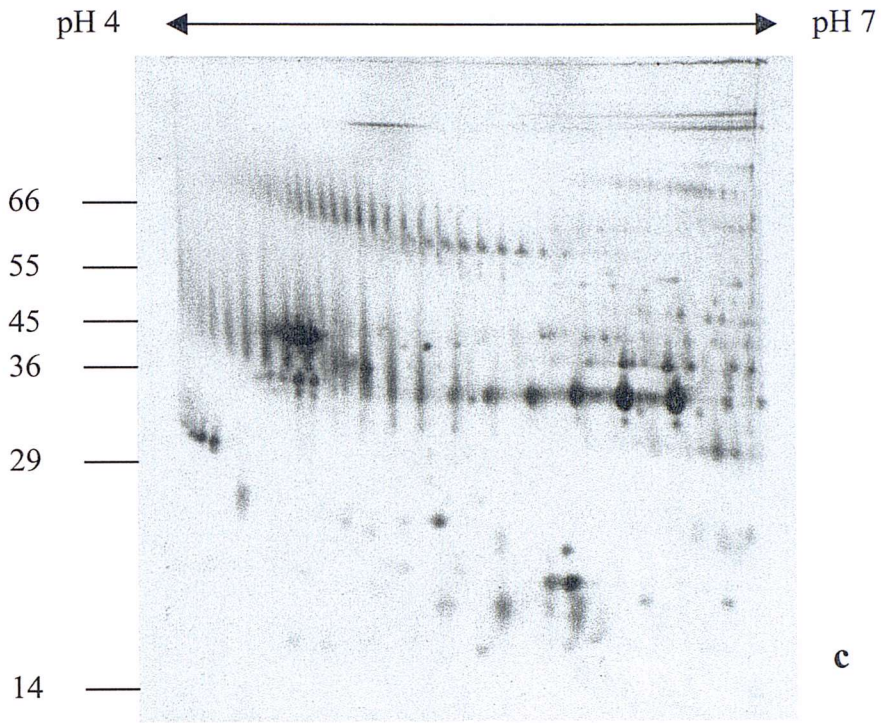


Fig 4.15. Silver stained 2D-gels (pH 4-7 gradient) of proteins from control (a) and IL-1 treated explant medium (b). Their corresponding autoradiographs are shown in (c) and (d) respectively. 3 g of porcine articular cartilage were treated without (a and c) or with IL-1α (b and d) for 20 h and then pulsed with [^{35}S]methionine/cysteine (200 μCi/ml) for 5 h. Explant medium was removed and treated with 3 mg CPC/mg GAG for 30 min at room temperature. The supernatant was removed and dialysed into water at room temperature and lyophilized. Protein samples were isoelectrically focused on a pH 4-7 gradient. Focused proteins were electrophoresed by SDS-PAGE on a 12.5 % gel. The gels were stained with silver, dried and exposed to film for 1 week. Proteins identified by ESI MS are labelled.



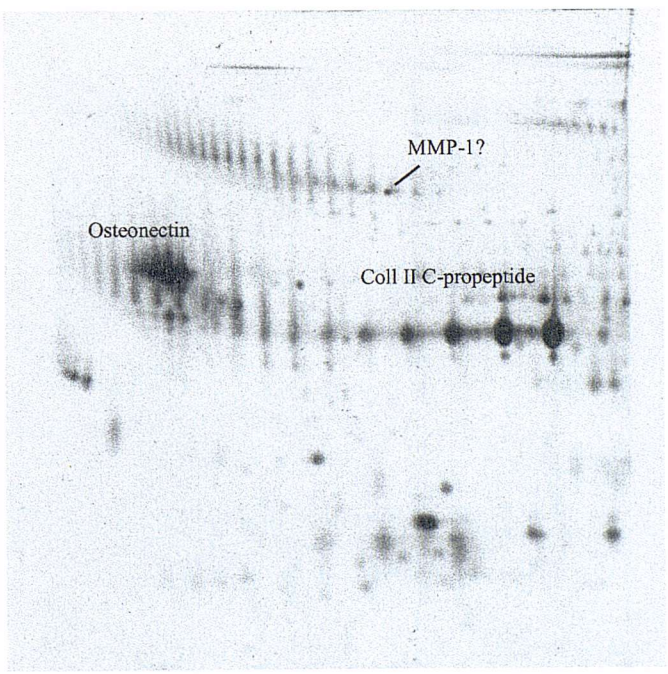


Fig 4.15 continued. Silver stained 2D-gels (pH 4-7 gradient) of proteins from control (a) and IL-1 treated explant medium (b). Their corresponding autoradiographs are shown in (c) and (d) respectively.

4.7 Discussion

Proteins secreted from porcine cartilage explants were studied by 2D-electrophoresis. Explant medium samples failed to isoelectrically focus. CPC precipitation solved the problem by removing polyanionic proteoglycans (mainly aggrecan) from the explant medium. 103 distinct proteins were identified from porcine explant medium including several well-known cartilage proteins (fig 4.13, table 4.1 and appendix 1). However only 7 or 8 of these were metabolically labelled and only 2 or 3 were IL-1 regulated.

Nevertheless at this stage because the focusing problem had been overcome, I went on to study human cartilage. Because this was my main aim: to compare proteins secreted from normal and osteoarthritic cartilage. The result of those studies is discussed in chapter 5.

But first I would like to describe some incomplete work, in which I studied proteins secreted from porcine chondrocytes in monolayer cultures by 2D-electrophoresis. A similar problem to the one experienced with explant medium samples occurred. The cell medium samples did not isoelectrically focus. I have chosen to present the data here as it is related to the work on explants.

Part II 2D-electrophoresis of porcine chondrocyte medium

4.8 Introduction

Proteins secreted from chondrocytes grown in monolayer culture also failed to isoelectrically focus (fig 4.16a). The sulphated GAG content in the medium was estimated to be less than 20 μg/ml, thus the amount of proteoglycans was much less than in the explant conditioned medium. CPC precipitation (3 mg CPC/mg GAG) of the sample failed to improve the isoelectric focusing of the proteins (fig 4.16b).

The focusing was not being prevented by aggrecan. Another candidate interfering polyanion produced by chondrocytes is hyaluronan. This very long GAG chain is composed of disaccharides of glucuronic acid and *N*-acetylglucosamine. Since it is non-sulphated, it is not detected by the DMB assay. The CEC for hyaluronan is 0.2 M (Prof. John Scott, personal communication).

This is similar to the CEC of culture medium (~0.15 M) and therefore CPC precipitation would work less efficiently on hyaluronan in cell medium. Concentrating the cell medium 100-fold made it very viscous, further indicating that there could be a high concentration of hyaluronan in the medium.

4.8.1 Hyaluronan removal by digestion with hyaluronidase

An experiment was set up to study if removal of hyaluronan would improve the 2D-gel pattern of cell medium. Hyaluronan was digested with hyaluronidase. This enzyme randomly cleaves beta-*N*-acetylhexosamine glycosidic bonds in hyaluronan, and chondroitin sulphates. 3 mg/ml hyaluronan was treated with different concentrations of hyaluronidase to determine the amount of enzyme to use for the cell medium. It was found that 150 units of hyaluronidase per 3 mg/ml hyaluronan was sufficient to remove enough viscosity to pass the material through a 22 micron filter of a centrifuge tube. Unconcentrated cell medium was not very viscous and therefore 15 units/ml and 150 units/ml was chosen for the experiment.

Cell medium was collected for 5 hours and incubated with either 15 units/ml or 150 units/ml of hyaluronidase for 30 min at 37°C. Samples were dialysed overnight at 4°C. After lyophilisation, the samples where 2D-electrophoresed and the gels stained with silver. More proteins were resolved of which especially the high molecular weight proteins were better focused compared to the control sample (fig 4.17). However, with the different concentrations of hyaluronidase used, it was clear that the enzyme preparation contained many proteins. Similarly to chondroitinase ABC lyase, this enzyme preparation was judged unsuitable for use in 2D analysis of complex mixtures of proteins.

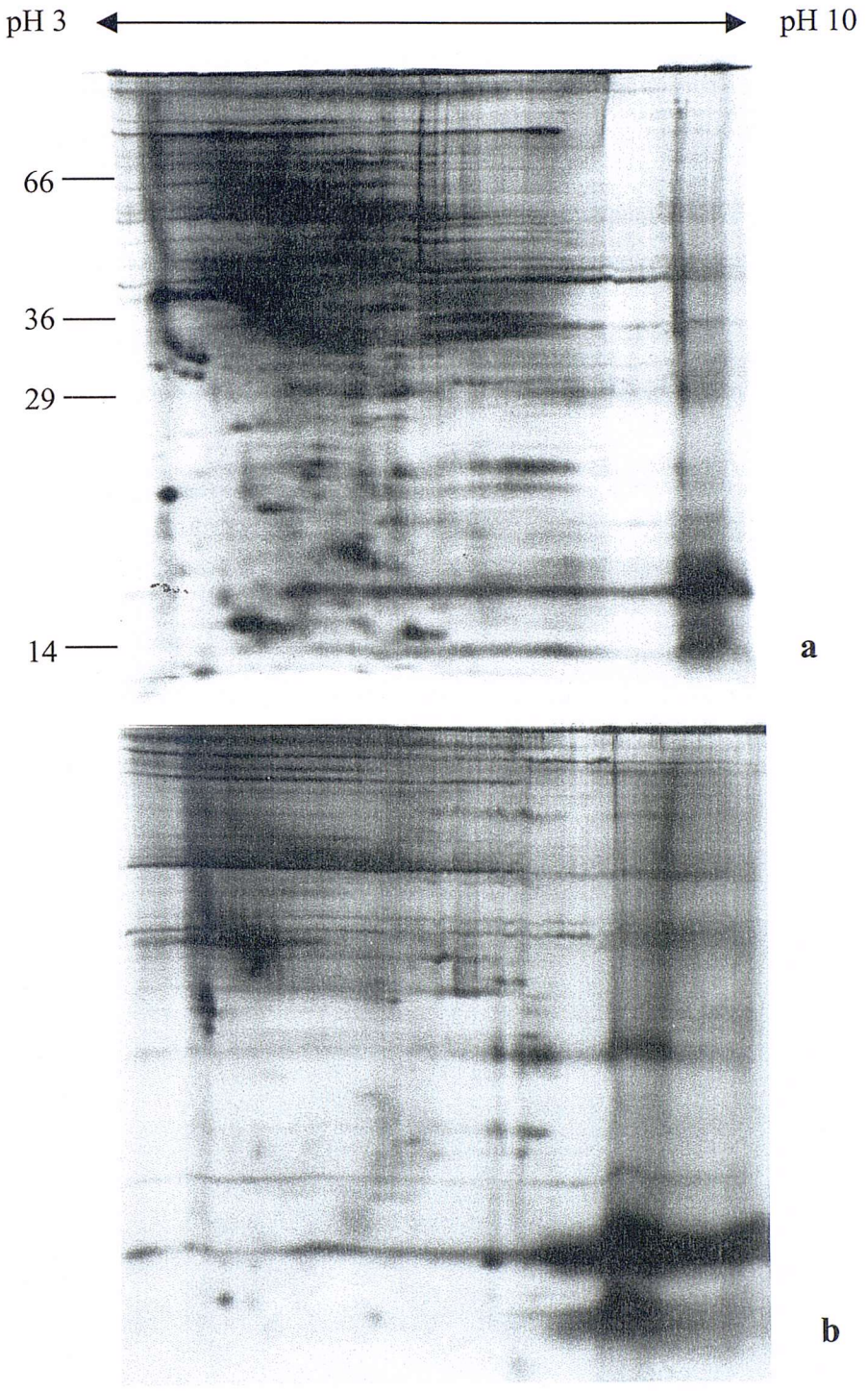


Fig 4.16. Autoradiographs of 2D-electrophoresis of cell medium without (a) and with CPC precipitation (b). 90 million cells were treated with IL-1 α (20 ng/ml) for 24 h and then pulsed with [^{35}S]methionine/cysteine (50 µCi/ml) for 5 h. The cell media were removed and treated with 3 mg CPC/mg GAG for 30 min at room temperature (b) or untreated (a). The supernatants were dialysed against water at 4°C and lyophilized. Approximately 100 µg of protein was used for isoelectric focusing on a 18 cm pH 3-10 gradient. Focused proteins were electrophoresed by SDS-PAGE on a 12.5 % gel. The gels were stained with silver and exposed to film for 5 days.

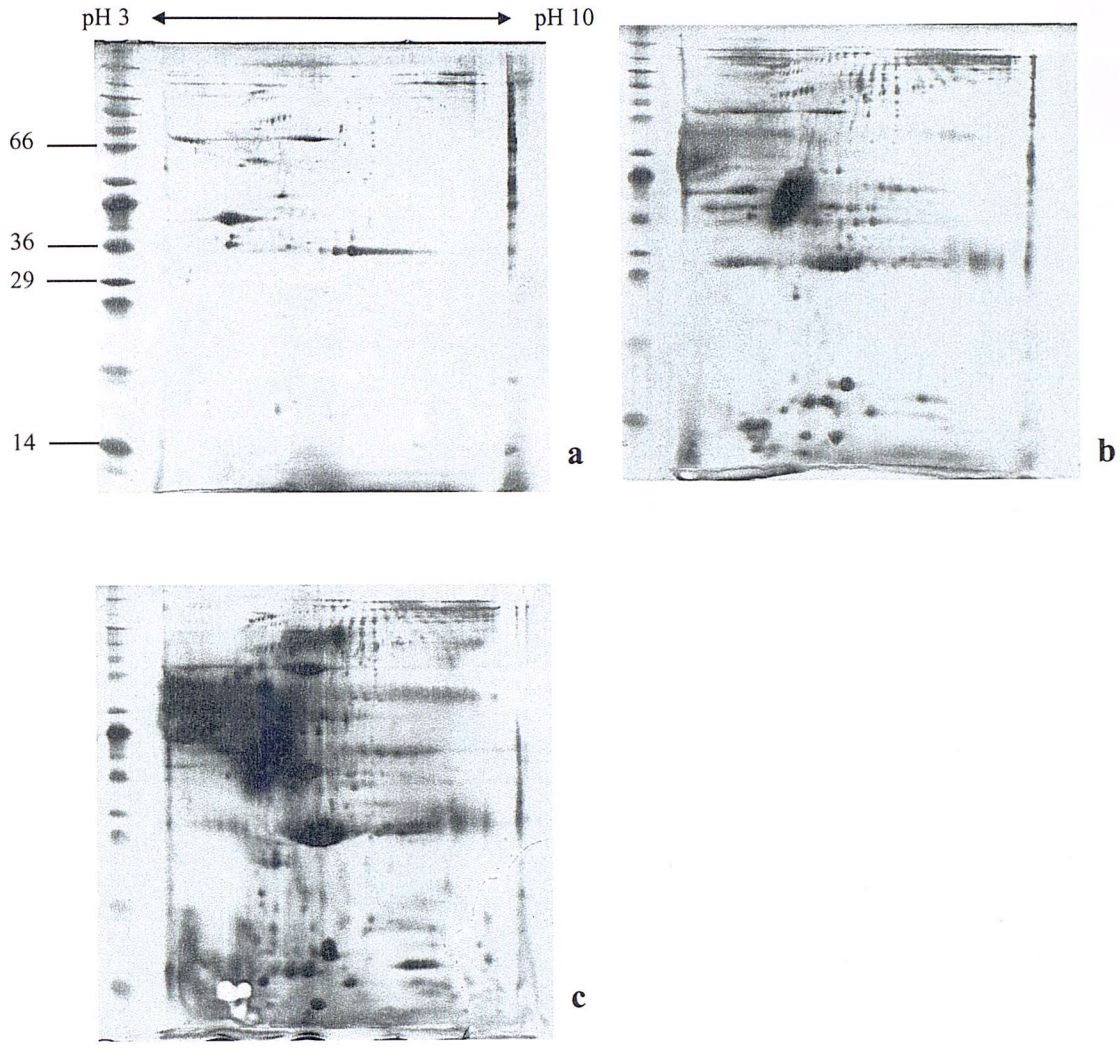


Fig 4.17. 2D-electrophoresis of chondrocyte media treated with different concentrations of hyaluronidase (b, c) or untreated (a). 35 million cells were serum starved overnight and then cell medium was conditioned for 5 h. The cell medium was removed and treated with 15 u/ml (b) or 150 u/ml (c) of hyaluronidase for 30 min at 37°C or not treated (a). All samples were dialysed into water at 4 °C and lyophilized. The samples were isoelectrically focused on a pH 3-10 gradient followed by electrophoresis by SDS-PAGE on a 12.5 % gel. Gels were stained with silver.

4.8.2 Use of an anion exchange column to remove hyaluronan

Despite the high content of impurities in the hyaluronidase preparation, the removal of hyaluronan seemed to improve the isoelectric focusing of proteins from cell medium. To investigate another method of removal, the cell medium was applied to an anion exchange column at pH 7.5. Under the conditions used the polyanionic hyaluronan should bind to the column while most proteins, other than acidic ones, run through. Some proteins however, may interact specifically with the hyaluronan and be lost using this approach. Hyaluronan has not been proved to be the cause of the focusing problem, which could also be due to another polyanion (e.g. nucleic acid) or a very negatively charged protein.

Cell medium was prepared as before and loaded onto an anion exchange column of pre-swollen microgranular diethylaminoethyl (DEAE) cellulose. The proteins that did not bind were collected. This run-through (RT) material and control cell medium were dialysed and lyophilized. Samples were 2D-electrophoresed and the gels stained with silver. The RT material produced a 2D-gel with 50-100 proteins resolved of which most were well focused (fig 4.18). More spots were observed after the medium was passed through an anion exchanger compared to control medium. Proteins that bound to the column were eluted with 0.5 M NaCl. This material will be analysed by SDS-PAGE in future studies.

At least two proteins were lost after the DEAE column treatment compared to control (fig 4.18 arrowed). In order to try to prevent these proteins binding to the column, the experiment was repeated with at pH 6.5. Cells were treated with IL-1 over night and metabolically labelled for 5 h. The pH of the cell medium was lowered and the sample applied to the DEAE column. RT material was collected and 2D-electrophoresed. At pH 6.5 the acidic proteins still bound to the column and some were metabolically labelled (fig 4.19 labelled). The labelled protein spot was identified as osteonectin. An unlabelled protein which was also lost binding to the DEAE column was identified as 14-3-3 protein epsilon (also known as protein kinase C inhibitor protein-1)(fig 4.19 a, marked with a star). 14-3-3 protein epsilon has been

described to be a regulator of cell signalling processes mediated by calmodulin-dependent protein kinase II and protein kinase C (Jin et al., 1996).

MMP-1, MMP-2 and MMP-3 were relatively abundant proteins secreted from cells stimulated with IL-1. 1D-analysis of cell medium had previously identified MMP-1 and MMP-3 by MS. The identification of MMP-2 (gelatinase A) was a new finding in this project. MMP-2 cleaves collagen fragments after initial cleavage by collagenases.

Labelled complement C3 was detected after DEAE column treatment of the medium. This was in agreement with previous 1D-analysis of proteins secreted from IL-1 stimulated cells. Gp-38 was detected as two large spots at the basic end of the gel. This was surprising since resolution of gp-38 in explant medium generally tended to be poor and variable on a pH 3-10 gradient. A row of labelled proteins had a typical TIMP-1 spot pattern. Identification of these was not possible since they were below the detection limit of silver staining and MS.

In order to try to improve protein recovery, especially of the acidic proteins the experiment will be performed with cell medium samples at pH 5.0 and in the presence of 200 mM salt. The experiment will be repeated with cell medium from cells treated with or without IL-1 to further analyse regulated proteins. Scaling up would identify more proteins.

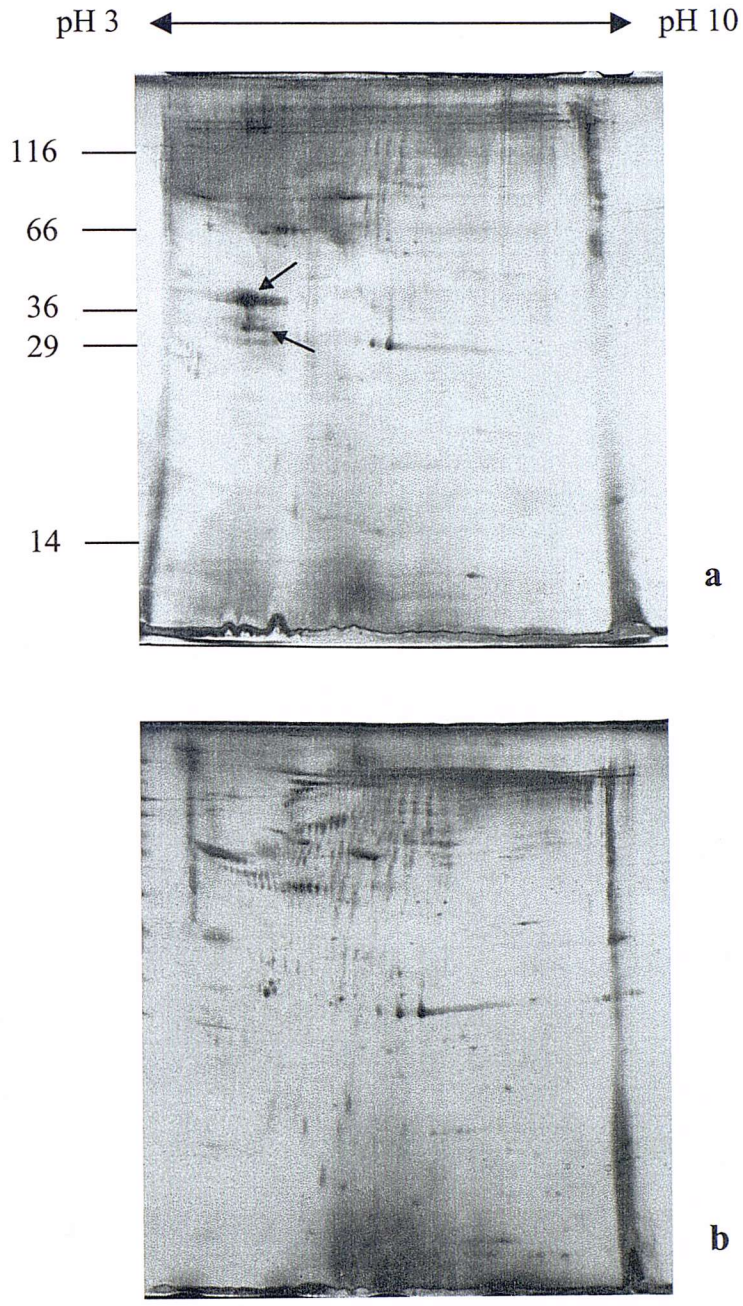


Fig 4.18. Silver stained 2D-gels of proteins secreted from chondrocytes before (a) and after DEAE column treatment (b). Chondrocytes were cultured in the absence of serum and medium was conditioned for 5 h. 16 ml of medium (from ~80 million cells) was collected. 8 ml of medium was prepared for 2D-electrophoresis directly (a). The pH of the other 8 ml of cell medium was lowered to 6.5 and it was passed through a column (10 cm x 1 cm) of DEAE cellulose. The column was pre-equilibrated with fresh DMEM (pH 6.5). Proteins that did not bind to the column were collected and prepared for 2D-electrophoresis (b). After dialysis and lyophilisation of the samples, the proteins were applied to a 13 cm IPG strip with a pH 3-10 gradient. Focused proteins were electrophoresed by SDS-PAGE on a 12.5 % gel. The gels were stained with silver. Proteins bound by the DEAE column are arrowed.

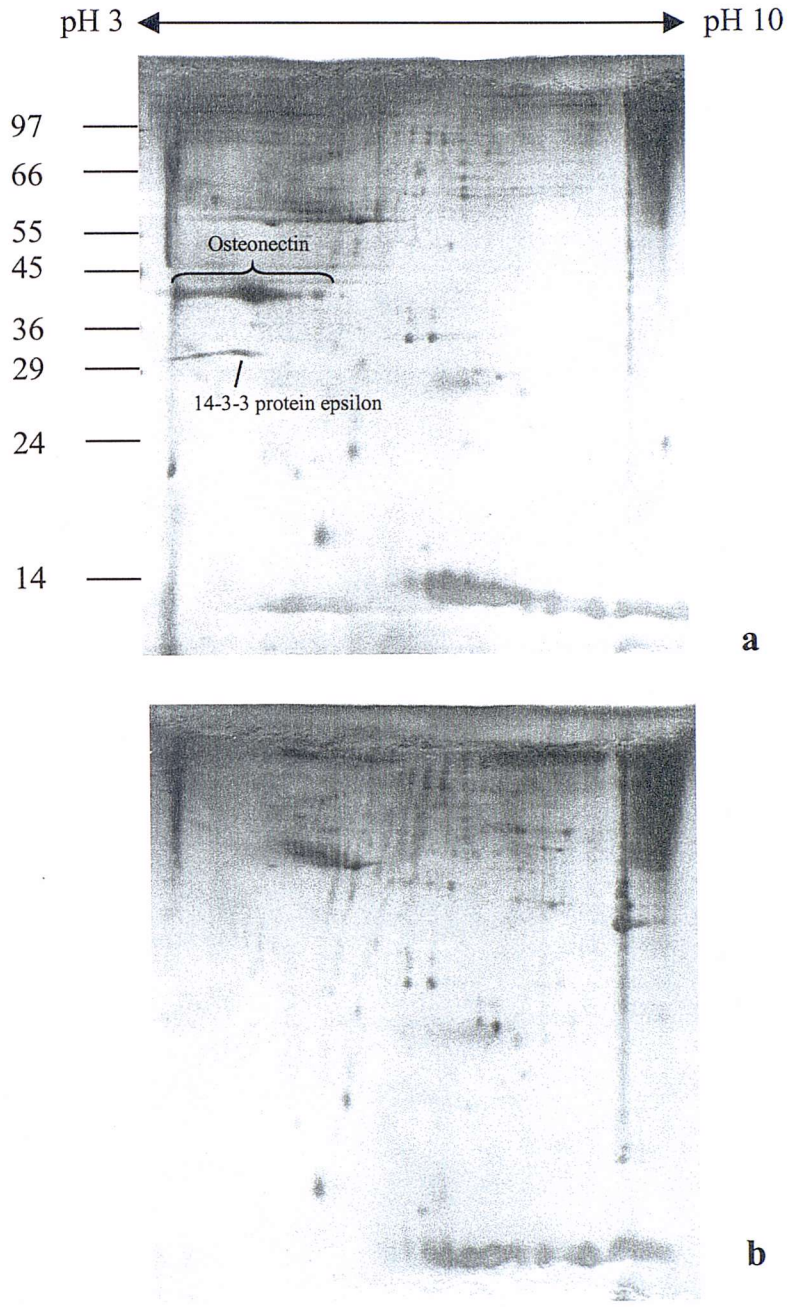


Fig 4.19. Silver stained 2D-gels of proteins secreted from chondrocytes stimulated with IL-1 before (a) and after DEAE column treatment (b). Their corresponding autoradiographs are shown in (c) and (d). Chondrocytes were treated with IL-1α (20 ng/ml) overnight and then pulsed with [^{35}S]methionine/cysteine (50 μCi/ml) for 5 h. The medium was removed and two samples prepared from it. One sample was prepared for 2D-electrophoresis directly (a and c). The pH was lowered to 6.5 for the other sample and it was passed through a column packed with DEAE cellulose. Proteins that did not bind to the column were collected and prepared for 2D-electrophoresis (b and d). After dialysis and lyophilisation of the samples, the proteins were applied to a 13 cm IPG strip with a pH 3-10 gradient. Focused proteins were electrophoresed by SDS-PAGE on a 12.5 % gel. The gels were stained with silver and exposed to film over night. Proteins were identified by MALDI and ESI MS.

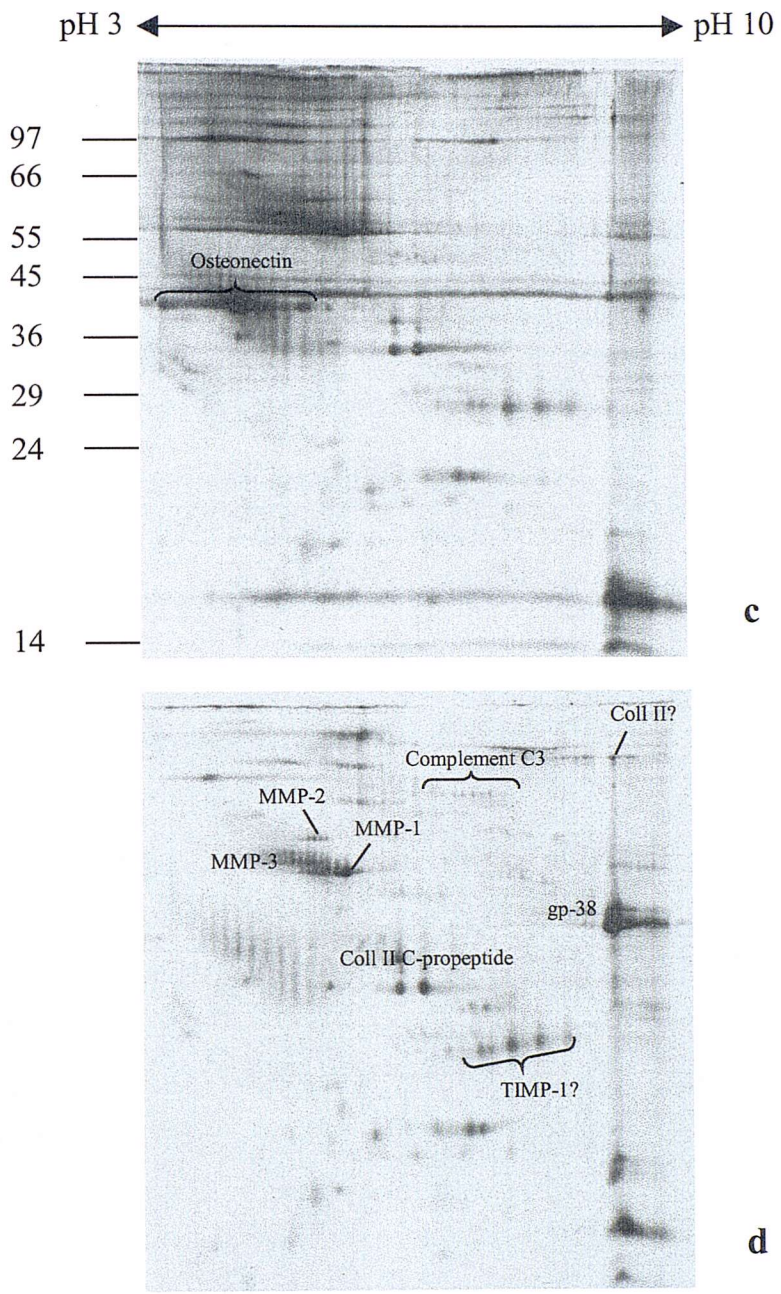


Fig 4.19 continued. Silver stained 2D-gels of proteins secreted from chondrocytes stimulated with IL-1 before (a) and after DEAE column treatment (b). Their corresponding autoradiographs are shown in (c) and (d).

4.9 Comparison of secreted proteins from cartilage explants and from chondrocytes grown in monolayer

The secreted protein pattern from unstimulated chondrocytes cultured as explants differs from chondrocytes cultured in monolayer (fig 3.1). This was further analysed by 2D-electrophoresis of secreted proteins. The 2D-map of explant medium from 6 g of cartilage (~ 100 million cells) was compared to the 2D-map of secreted proteins from approximately 40 million cells grown in monolayer (fig 4.20). In both cases the chondrocytes were stimulated with IL-1. The main labelled proteins secreted by chondrocytes in explants and monolayer cultures were the α-chain and C-propeptide of collagen type II, osteonectin and TIMP-1 (fig 4-19 and 4-20). More labelled protein was secreted from explants than from cells in monolayer, but this was probably because of the larger number of cells in the explant experiment. When the protein recovery of cell medium from the DEAE column has been optimised, a scaled-up experiment will be performed for better comparison including medium from unstimulated monolayer cells.

MMP-1 and gp-38 were induced by IL-1 in both types of culture. The induction was much stronger in the cell medium samples. It is possible that the release of MMP-1 and gp-38 from explants is hindered or delayed by the extracellular matrix. MMP-2 and MMP-3 were secreted from cells grown in monolayer. IL-1 upregulated MMP-3 was previously identified by 1D-analysis of explant medium and cell medium. MMP-2 had not been identified in the 1D experiments.

TIMP-1 was upregulated by IL-1 in monolayer cultures (*chapter 3*). However in explant cultures both control and IL-1-treated explants secreted large amounts of TIMP-1. Complement C3 was identified from cell medium but not from explant medium. It is possible that complement C3 is upregulated in explants as well but has not focused on the 2D-gel. Labelled collagen type VI was identified from the cell medium sample, but was not found in the explant medium sample. Type VI collagen is known to bind several matrix molecules like collagen type II and XIV, decorin and hyaluronan (Pullig et al., 1999). Collagen type VI may interact with large molecules in the matrix and therefore was not detected in the explant medium sample.

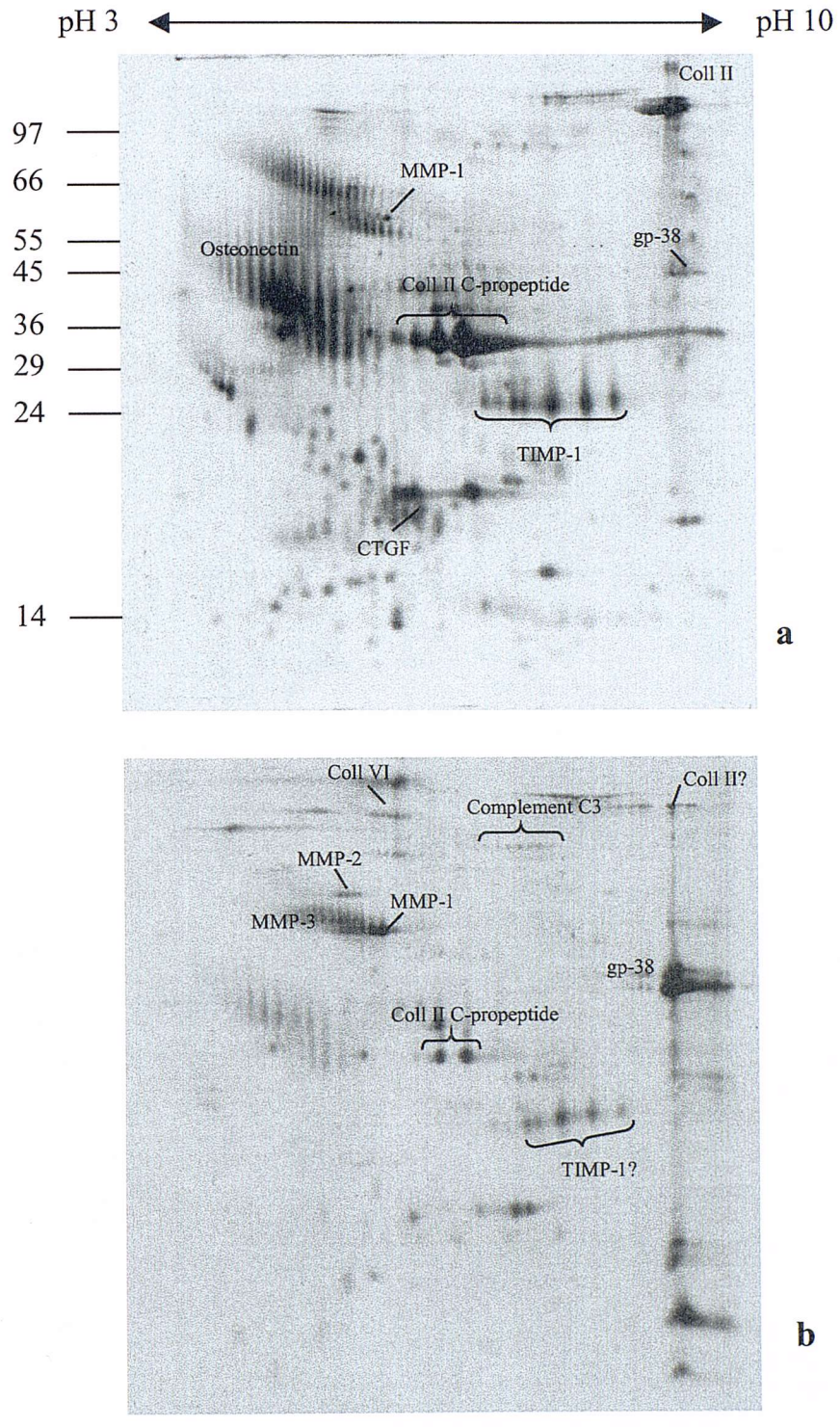


Fig 4.20. Comparison of secreted proteins from cartilage explants (a) and chondrocytes cultured in monolayer (b). 2D-gels were prepared as described in fig 4.12 and fig 4.19.

4.10 Discussion

Proteins secreted from cells grown in monolayer failed to isoelectrically focus. This was probably due to the presence of hyaluronan secreted by the cells. This theory has not yet been confirmed, but removal of polyanionic molecules by passing the medium through an anion exchange column greatly improved the focusing. Osteonectin and 14-3-3 protein epsilon bound to the column. Whether this binding was due to the proteins acidic pI (4.66 and 4.63 respectively) or because they specifically interacted with hyaluronan or another polyanionic molecule is not known. Further work will be done to improve the protein recovery from the DEAE column by decreasing the pH and increasing the salt concentration.

4.10.1 Comparison of protein identification by 1D- or 2D-electrophoresis

Many more proteins were detected and identified by 2D-electrophoresis due to higher protein loading and better separation of proteins. However more IL-1 regulated proteins were identified from a 1D-gel (*chapter 3*). Some of these were also detected by 2D-electrophoresis. Upregulated MMP-1 and gp-38 were detected in explant medium. MMP-3 was detected as a non-labelled spot but it is presumably focusing as several spots (as in the cell medium sample, see fig 4.19), which makes identification difficult in the presence of co-migrating proteins (fig 4.12b and d). TIMP-1 was also detected in explant medium but was not regulated by IL-1 in contrast to 1D-analysis of monolayer cell medium (fig 4.12 c and d). Further work need to be performed to determine whether the TIMP-1 upregulation is dependent on culture conditions.

In cell medium MMP-1, MMP-2 and MMP-3 were detected by 2D-analysis. MMP-2 was not previously detected by 1- or 2D-electrophoresis. This enzyme is unique in that it is constitutively expressed and not upregulated by IL-1. It lacks the propeptide sequence other MMPs have that is susceptible to proteolytic activation. Activation of pro-MMP-2 occurs on the cell membrane via cleavage by membrane-type 1-MMP (MT1-MMP) (Sato et al., 1996) or directly by activated protein C (Nguyen et al., 2000). Complement C3 was detected in cell medium by 2D-

electrophoresis but not in explant medium. 1D-analysis of concentrated explant medium will be performed to establish whether this was due to different culture conditions or because complement C3 does not focus on a 2D-gel. The labelled proteins exhibiting a typical TIMP-1 like pattern in the cell medium sample will be identified by scaling up the experiment.

Generally, the analysis could be improved with a higher protein loading and by the use of different pH gradients to "zoom" in on specific pI- ranges.

However, the technique was not further pursued with porcine chondrocytes.

4.10.2 Application of developed methods to study chondrocytes protein expression

In conclusion, methods were developed to study proteins secreted from chondrocytes in cartilage explants and from monolayer cultures. These methods can be applied to study chondrocyte protein expression in different environments, with different stimuli and in different types and regions of cartilage. They can be used to compare protein synthesis in normal and diseased cartilage and to study the effects of loading on cartilage. In the following chapter, proteins secreted from normal and OA articular cartilage explants were compared.

Chapter 5

Comparison of proteins secreted by chondrocytes in osteoarthritic and normal cartilage

5 Introduction

The histological features of OA were described in chapter 1. Such visible differences suggest that there will be significant differences in protein expression between normal and OA articular cartilage. One way to investigate this is to use a proteomic approach to define the proteins secreted by chondrocytes in explant culture. Identification of proteins strongly up- or down-regulated by chondrocytes in OA cartilage could provide important information about the pathogenesis of these. Changes in regulatory molecules could shed light on the basic mechanisms. Changes could also lead to identifications of markers for OA. In this chapter the techniques developed to study proteins secreted by porcine explants (*chapter 4*) were applied to human cartilage.

5.1 Obtaining cartilage specimens

Ten human articular OA cartilage specimens were compared to thirteen normal human articular cartilage controls. Cartilage was obtained with informed consent from patients undergoing joint replacement surgery or amputations at the Charing Cross Hospital in Hammersmith, London. Some normal cartilage specimens came from post mortem autopsies in Ghent and Bruges in Belgium. All tissue specimens analysed are listed in table 5.1.

The amount of cartilage obtained from human specimens varied. Generally, at least 1.5 g of tissue was needed to produce 2D-gels containing sufficient amounts of protein to obtain mass spectrometric data from metabolically labelled components. The data shown came from tissue samples varying from 1.0-6.2 g. Many of the OA specimens received were removed inevitably at a very late stage of the disease at which most, if not all cartilage was already lost. Such heavily denuded specimens were of no use for our purpose and were discarded. Normal healthy cartilage specimens were hard to obtain. Three of the normal specimens came from amputations for conditions, which may have affected the cartilage. These were

cartilage from a humeral head with an adjacent metastatic breast carcinoma (N10), cartilage from an ankle joint of a limb with osteomyelitis (N11), and from a humeral head excised for avascular necrosis (N12) (see table 5.1).

Table 5.1 List of human cartilage specimens used for proteomic analysis

Sample	Diagnosis	Tissue type	Tissue (g)	Sex	Age	Source
N1	normal	ankle joint	3.5	m	22	CX*
N2	normal	femoral head	3.0	f	82	CX
N3	normal	femoral head	2.7	f	86	CX
N4	normal	femoral head	3.0	f	78	CX
N5	normal	femoral head	3.0	f	70	CX
N6	normal	femoral condyle	3.4	?	49	Ghent
N7	normal	femoral condyle	2.7	m	65	Bruges
N8	normal	knee joint	?	?	?	Bruges
N9	normal	ankle joint	?	?	?	CX
N10	normal[1]	humeral head	2.2	f	60	CX
N11	normal[2]	ankle joint	5	f	?	CX
N12	normal[3]	humeral head	2.2	m	32	CX
N13	normal	femoral condyle	?	?	7 weeks	Ghent
OA1	OA	humeral head	1.8	f	76	CX
OA2	OA	femoral head	1.0	f	54	CX
OA3	OA	femoral head	1.7	f	70	CX
OA4	OA	femoral head	2.7	f	63	CX
OA5	OA	femoral condyle	3.0	m	65	CX
OA6	OA	?	1.5	f	62	CX
OA7	OA	femoral condyle	6.2	f	53	CX
OA8	OA	femoral condyle	2.3	f	83	CX
OA9	OA	femoral condyle	2.0	m	60	CX
OA10	OA	humeral head	2.2	f	72	CX

* tissue obtained from Charing Cross Hospital

[1] patient diagnosed with metastatic breast cancer

[2] amputation due to osteomyelitis

[3] excision following avascular necrosis

These cartilage specimens looked healthy but it was uncertain to what extent the disease could have affected the tissue. Due to the limited numbers, it was not possible to age and sex match the cartilage samples.

5.2 2D-electrophoresis of human explant medium samples

Cartilage was dissected from the bone, washed once and left overnight in serum-free DMEM to wash out plasma proteins and other non-chondrocyte proteins. Next day, the tissue was pulsed with [^{35}S]methionine/cysteine for 5 h. The supernatant was collected and the GAG content estimated with the DMB-assay. Proteoglycans were removed by precipitation with 3 mg CPC/mg GAG (the concentration found to be optimal for removing proteoglycans from porcine explant medium see chapter 4). The supernatant was dialysed into water at 4°C or room temperature over-night. After lyophilization the sample was run in 2D-electrophoresis and the gel stained with silver. About 200-300 protein spots per gel were resolved of which most were well focused (fig 5.1). Fifty to 100 protein spots were metabolically labelled. Eighteen labelled proteins were identified by MS analysis (fig 5.2). These identifications were mainly done from OA sample 1 (OA1). The pattern of the major metabolically labelled proteins of human explant medium samples was surprisingly reproducible. A comparison of labelled proteins secreted by OA or normal cartilage explants are shown in fig 5.3. All autoradiographs of human explant medium samples used for analysis are shown in fig 5.4 (normal) and fig 5.5 (OA). Nine of the gels were prepared by Dr Mark Bolton.

Very little C-propeptide or α-chain of collagen type II were synthesised by the human samples compared with the amount produced by the porcine explants (see e.g. fig 5.2). This was probably because of the difference in relative age between adolescent pigs and adult humans.

Two groups of labelled proteins in the acidic part of the gel focused as sets of adjacent spots that trailed badly in the SDS dimension. The higher molecular weight group was identified as lumican. This keratan sulphate proteglycan appears

predominantly in a glycoprotein form in adult cartilage (Grover et al., 1995). The heterogeneity could be due to varying glycosylation.

The smaller group of acidic proteins were identified as clusterin, a heterodimeric glycoprotein found in many tissues. Clusterin is induced in response to tissue injury (Connor et al., 2001) and may play a protective role in cells under stress (Viard et al., 1999).

MMP-2 was identified from a well-focused protein spot above the lumican cluster and MMP-3 was detected within the lumican cluster. These MMPs are known to play a role in matrix degradation and have previously been detected in OA cartilage and synovial fluid (Tetlow et al., 2001; Yoshihara et al., 2000). MMP-1 was not detected on the 2D-gel but it possible it is also hidden in the cluster of lumican since it was identified from an 1D-gel (see fig 3.23). The amount of the MMPs, especially MMP-3 was impossible to estimate since it co-migrated with lumican. This is a good example of the limitations of the 2D approach for protein quantification. No MMPs were identified in the normal cartilage samples.

A set of five spots corresponding to a protein of about 30 kDa were always strongly labelled. They were identified as tissue inhibitor of metalloproteinases-1 (TIMP-1) and were found on all 2D-gels of human explant medium. TIMPs are important regulators of MMPs that participate in the degradation of the extracellular matrix. Because of the consistency of the TIMP spots throughout all the samples, the central one was used for normalising the relative amounts of other protein spots, which varied between different 2D-gels. It could be argued that TIMP-1 is a regulated molecule, at least in porcine cartilage (e.g. by IL-1, bFGF see *chapter 3/4*) and not suitable for this purpose. However, the TIMP-1 spots always focused and electrophoresed sharply and were a very constant feature of the metabolic labelling pattern (fig 5.4 and fig 5.5).

Labelled TIMP-2 was detected on most gels (normal and OA) as two weak spots below TIMP-1.

Osteonectin (SPARC) was another abundant protein synthesised by human chondrocytes. Its function in cartilage is unclear but it has been suggested, that it play

roles in cell differentiation, cell cycle modulation and regulation of growth factor activity. (Chandrasekhar et al., 1994).

ASPIC (*A*cidic *s*ecreted *p*rotein *i*n *c*artilage) is a novel protein that our group identified by MS analysis of 2D-electrophoresed human explant medium. The protein shares no significant similarities or known protein motifs with other protein families except for an EGF-like domain. In 2001 Steck et al reported this protein as chondrocyte expressed protein-68 (CEP-68), and suggested it could be used as a marker for distinguishing chondrocytes from osteoblasts and mesenchymal stem cells in culture. Expression of CEP-68 mRNA was detected in cartilage, bone, lung and a smaller transcript in brain.

Another protein which was secreted in relative abundance by the explant chondrocytes was type VI collagen. This is a minor collagen in cartilage that forms its own fibrillar network. Its function is unclear. It binds to matrix proteins such as decorin, fibromodulin, fibronectin, other collagens and also to hyaluronan. Therefore it has been suggested to act as an interface between the rigid matrix and the chondrocytes in cell anchoring and signalling (Soder et al., 2002).

YKL-39 belongs to the chitinase family, but does not possess chitinolytic activity. Expression of YKL-39 has been found mainly in chondrocytes and to a lesser extent in synoviocytes, lung and heart (Hu et al., 1996). It is related to YKL-40 (49 % amino acid identity) and the two proteins may have similar functions, but these are currently unknown. YKL-39 was a constant feature of the human explant medium pattern. Labelled YKL-40/gp-39 was not always detected.

Labelled cytokine-like protein C17 was identified as a small protein spot around 20 kDa. No homology to this protein was found in the public databases when it was first identified (Liu et al., 2000). Analysis of the primary structure predicted that the protein contained four α-helices, a characteristic of hematopoietic cytokines and interleukins. Therefore the protein was named cytokine-like protein C17.

Connective tissue growth factor (CTGF) was sometimes detected as a labelled protein spot about 20 kDa in size (fig 5.3). Sequencing of human cartilage cDNA libraries have suggested that the CTGF is one of the most abundantly expressed genes in both normal and OA articular cartilage (Kumar et al., 2001).

The low abundance of these smaller proteins made it difficult to identify them on all 2D-gels analysed.

Labelled intact COMP was found on some gels (e.g. fig 5.3a), but was more often present as smaller unlabelled fragments.

Other proteins expressed by chondrocytes included complement factor B and procollagen C-proteinase enhancer protein. The latter binds to the C-terminal propeptide of type I-III procollagen and enhances the activity of procollagen C-proteinase (Hojima et al., 1985).

As with porcine explant media, most of the stained proteins were not labelled. MALDI and ESI MS/MS identified 92 different proteins or protein subunits from the OA cartilage explant medium sample (fig 5.1, table 5.2). The peptide sequences upon which the identifications were based are all given in Appendix 2.

Some unlabelled matrix proteins were identified including fibromodulin and cartilage intermediate layer protein (CILP). However it was not possible to know if they were chondrocyte derived.

In order to identify additional newly synthesised proteins, pooling of several different tissue samples would be necessary to increase the total protein loading.

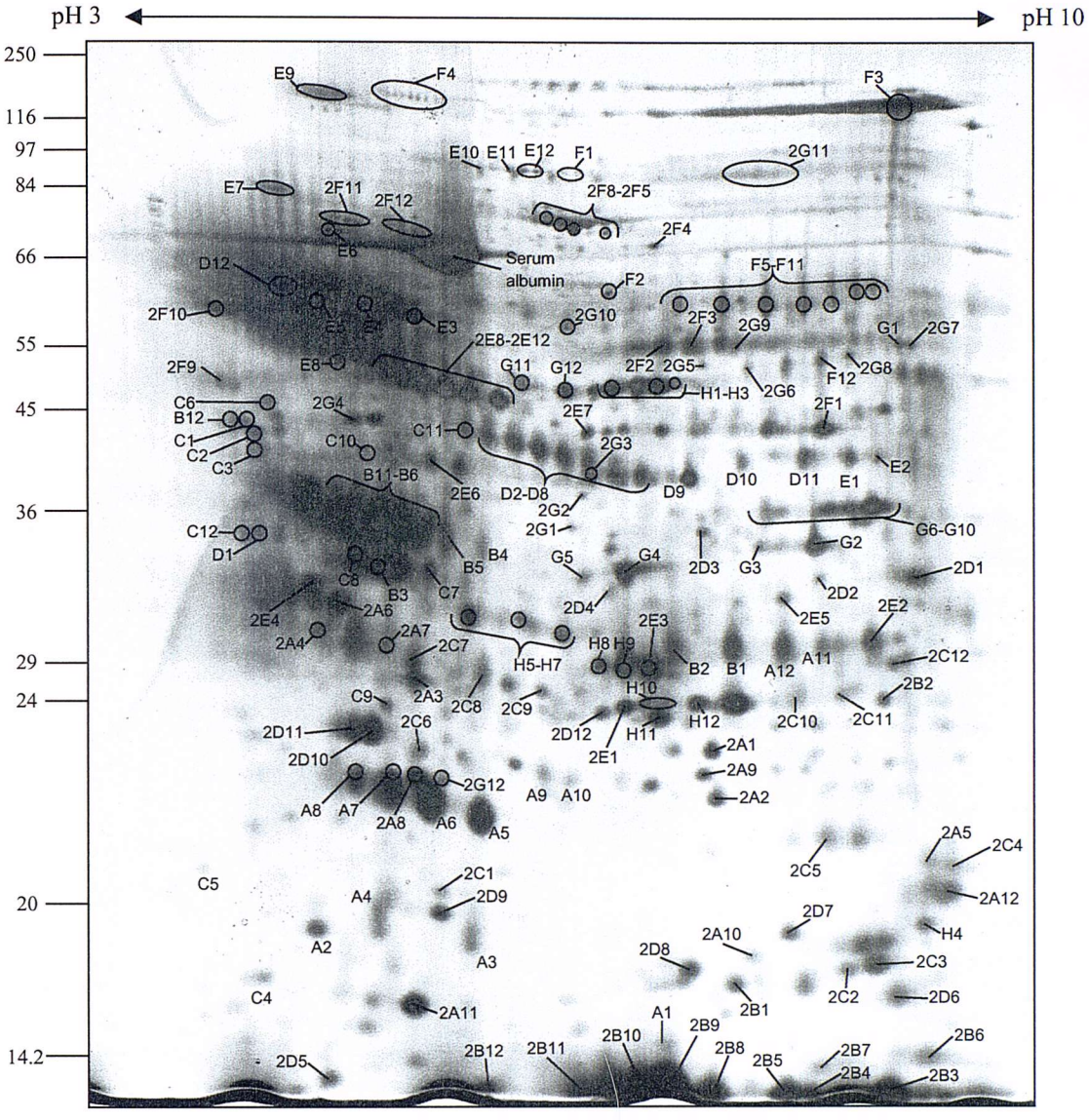


Fig 5.1. Silver stained 2D-gel of proteins secreted from OA cartilage explant medium (OA1). The corresponding autoradiograph is shown in fig 5.2. 1.8 g of cartilage was removed from a humeral head and washed overnight in serum free DMEM. The following day the cartilage was pulsed with [^{35}S]methionine/cysteine (200 µCi/ml) for 5 h. The explant medium was removed and treated with 3 mg CPC/mg GAG for 30 min at room temperature. The supernatant was removed and dialysed into water over night at 4°C and lyophilized. 60 µg of protein was used for isoelectric focusing on a pH 3-10 gradient. Focused proteins were further separated by SDS-PAGE on a 12.5 % gel. The gel was stained with silver, dried and exposed to film for 1 week. The gel was rehydrated and protein spots excised. Proteins were identified by MALDI and ESI MS. Identified proteins corresponding to the labels on the gel are listed in table 5.2.

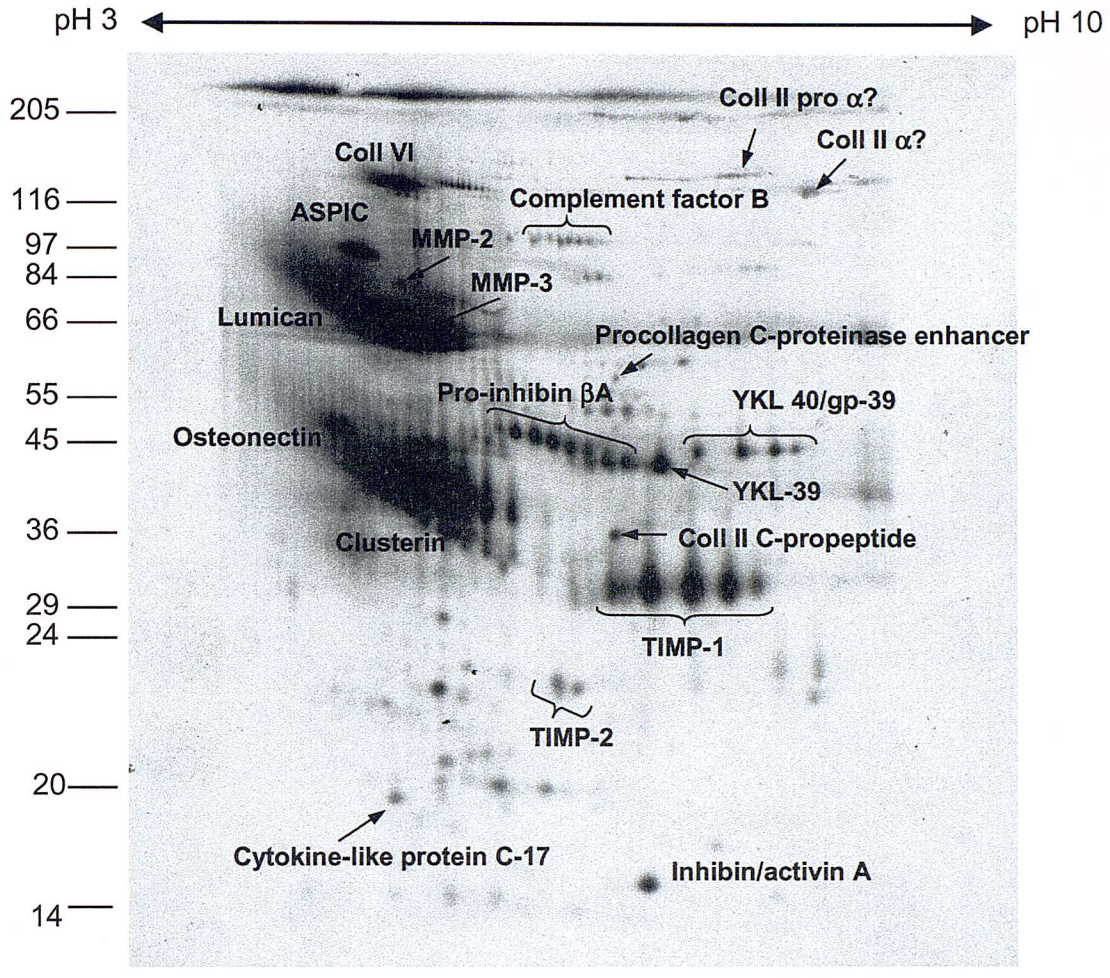


Fig 5.2. Autoradiograph of silver stained gel in fig 5.1 of OA cartilage explant medium (OA1). Collagen pro- α- and α-chains were not identified by MS from this particular sample, but due to the similarity in spot pattern they are labelled here. Abbreviations are defined in the text.

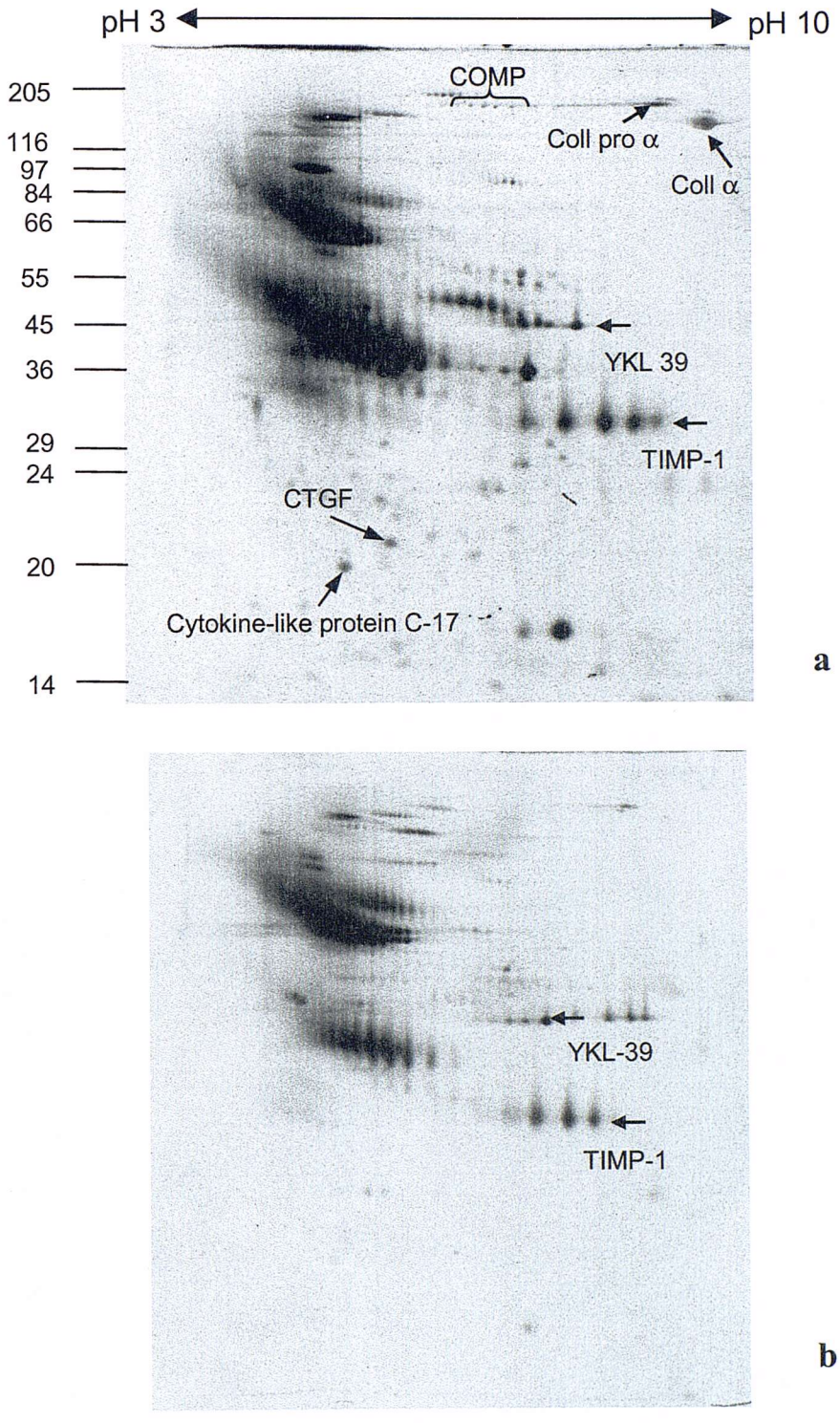


Fig 5.3. Comparison of labelled proteins secreted by OA (OA10) (a) or normal (N9) (b) cartilage explants. Gels prepared as described in fig 5.1. Abbreviations are defined in the text.

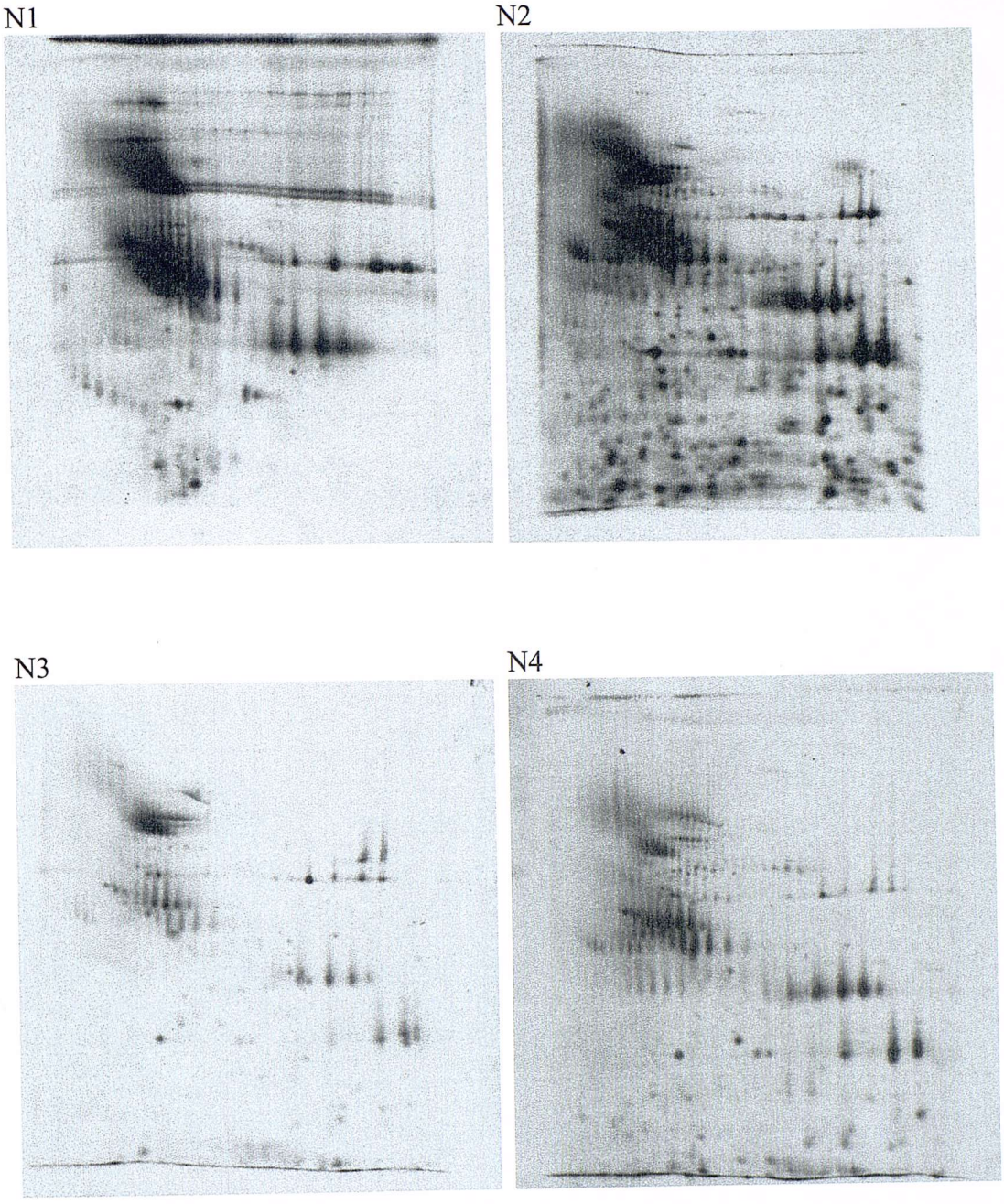


Fig 5.4. Autoradiographs of normal human explant media samples N1-N13.

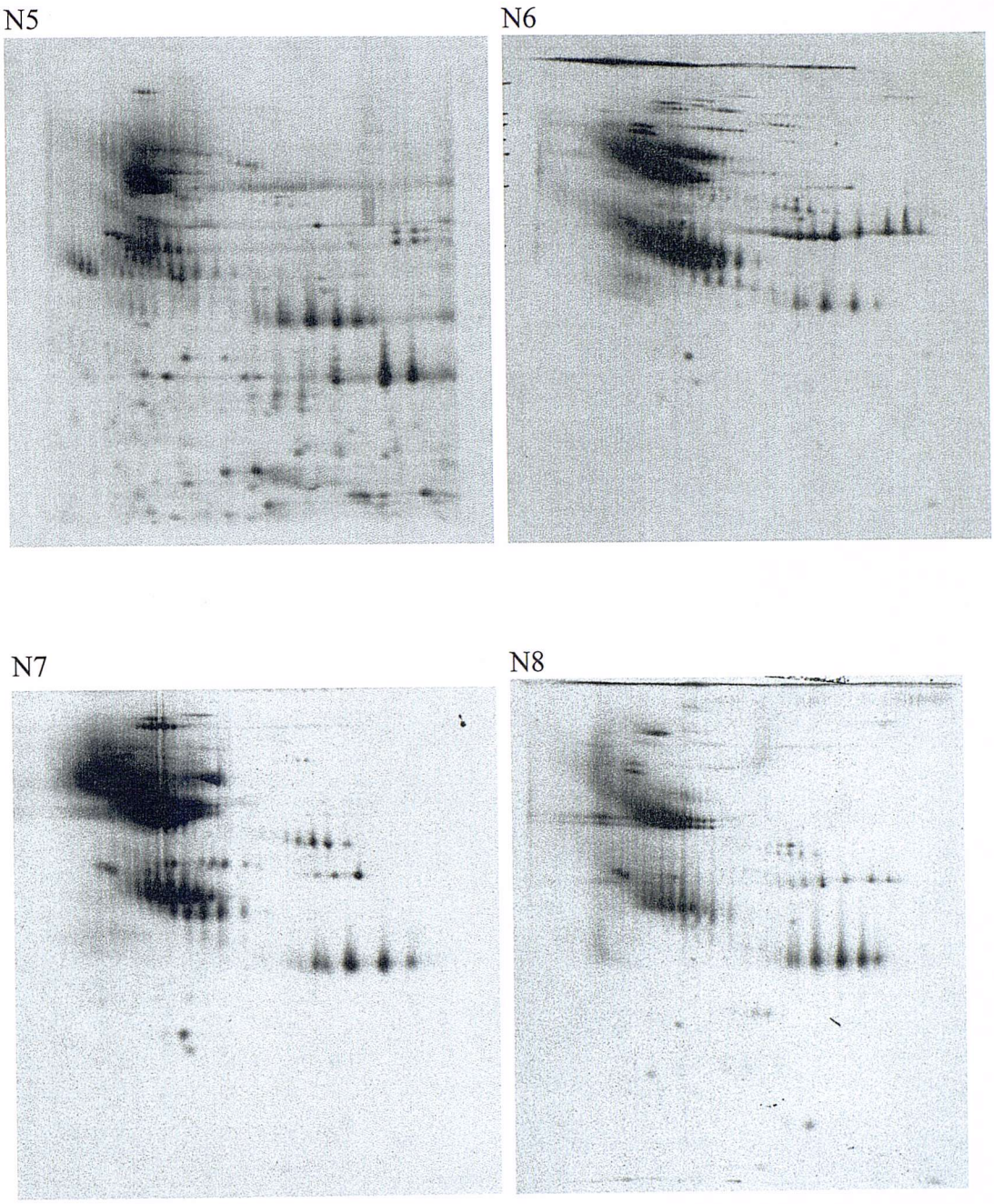


Fig 5.4. Autoradiographs of normal human explant media samples N1-N13.

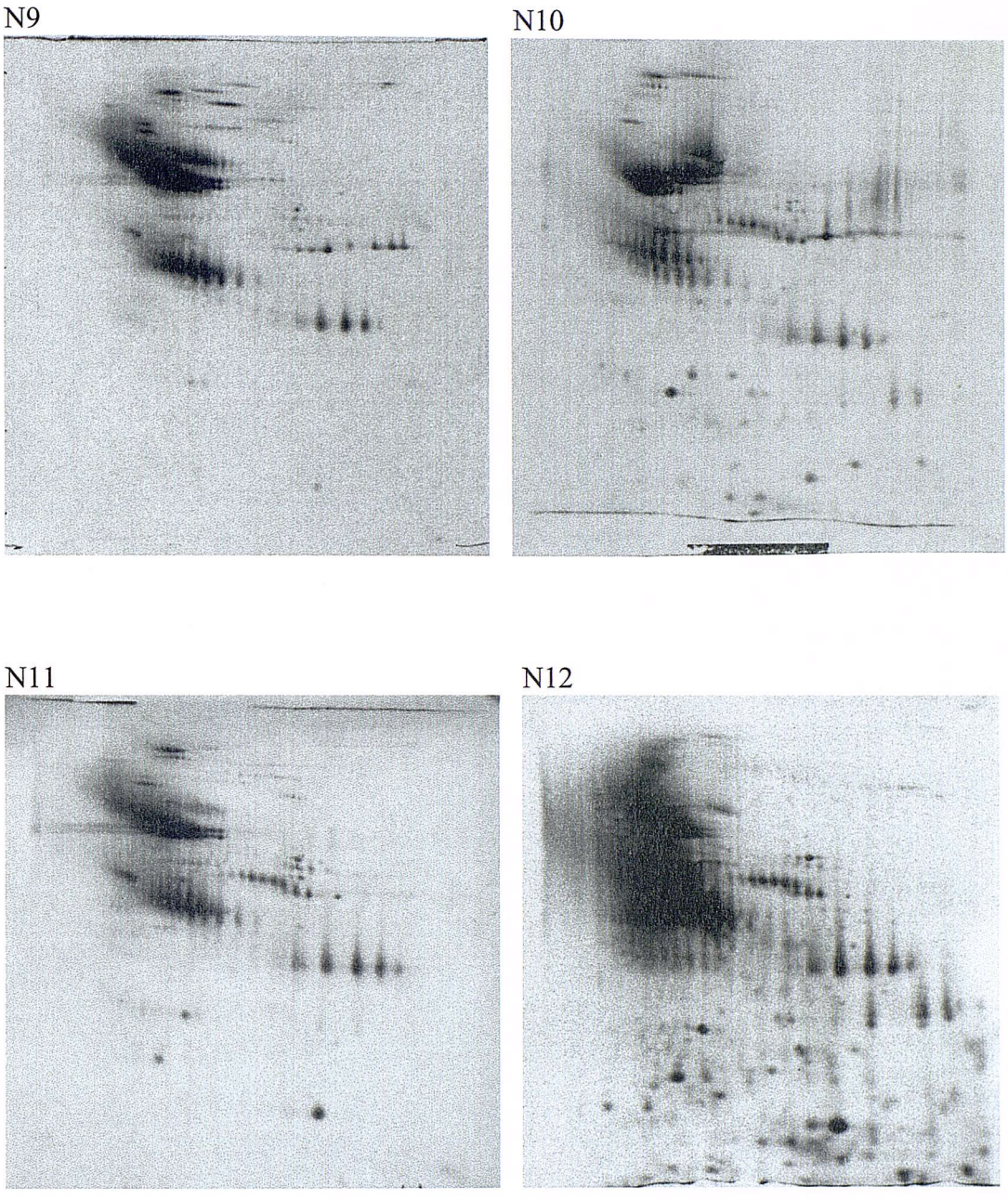


Fig 5.4. Autoradiographs of normal human explant media samples N1-N13.

N13

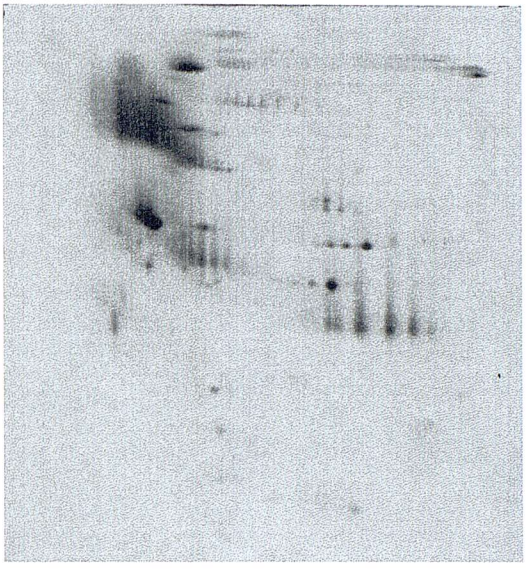
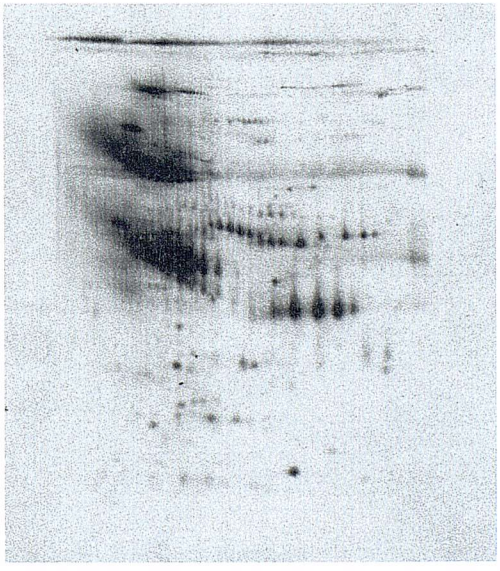


Fig 5.4. Autoradiographs of normal human explant media samples N1-N13.

OA1



OA2

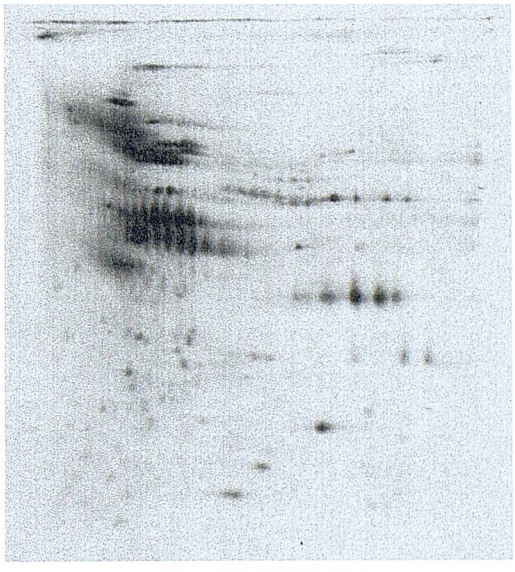


Fig 5.5. Autoradiographs of OA human explant media samples OA1-OA10.

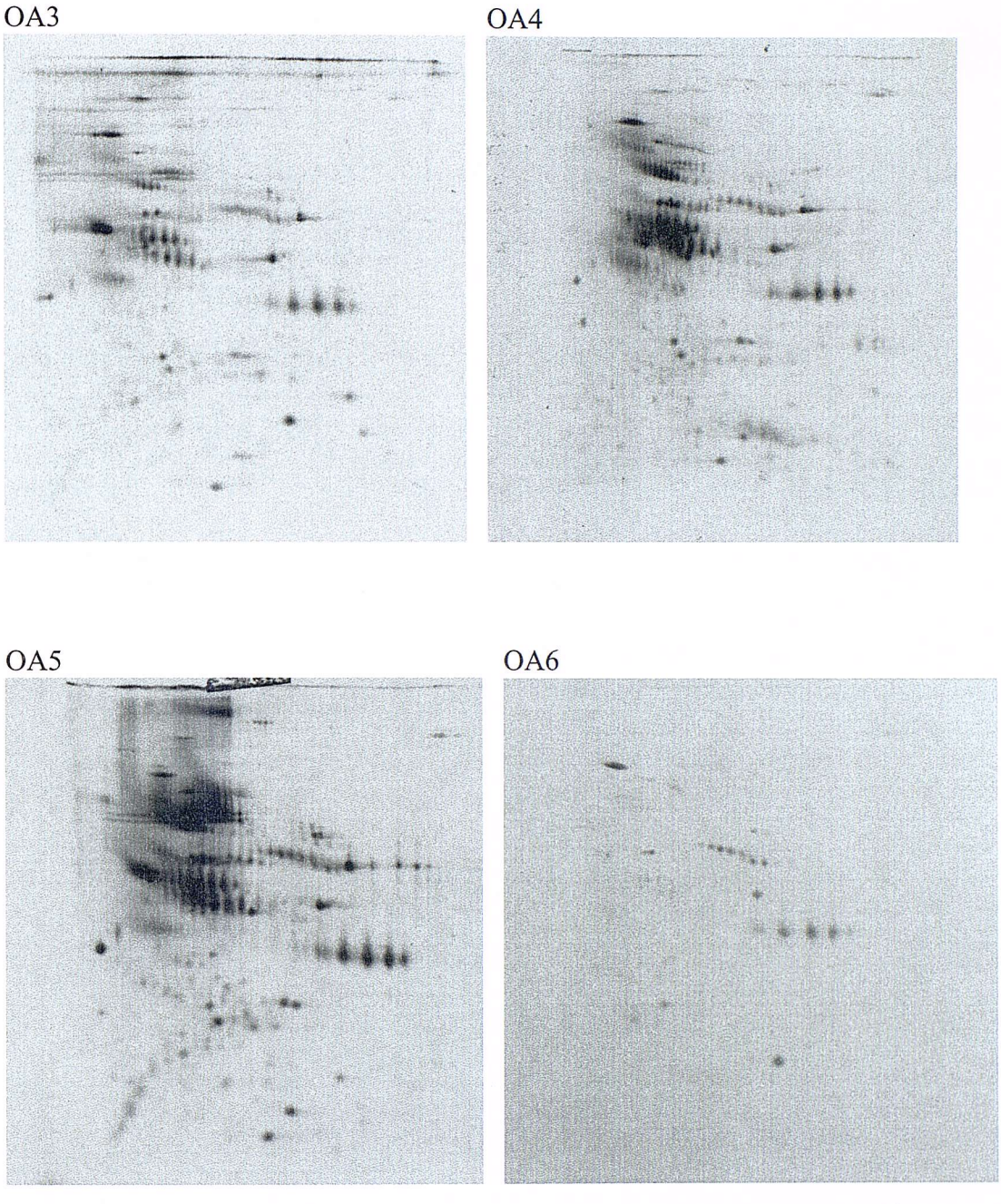


Fig 5.5. Autoradiographs of OA human explant media samples OA1-OA10.

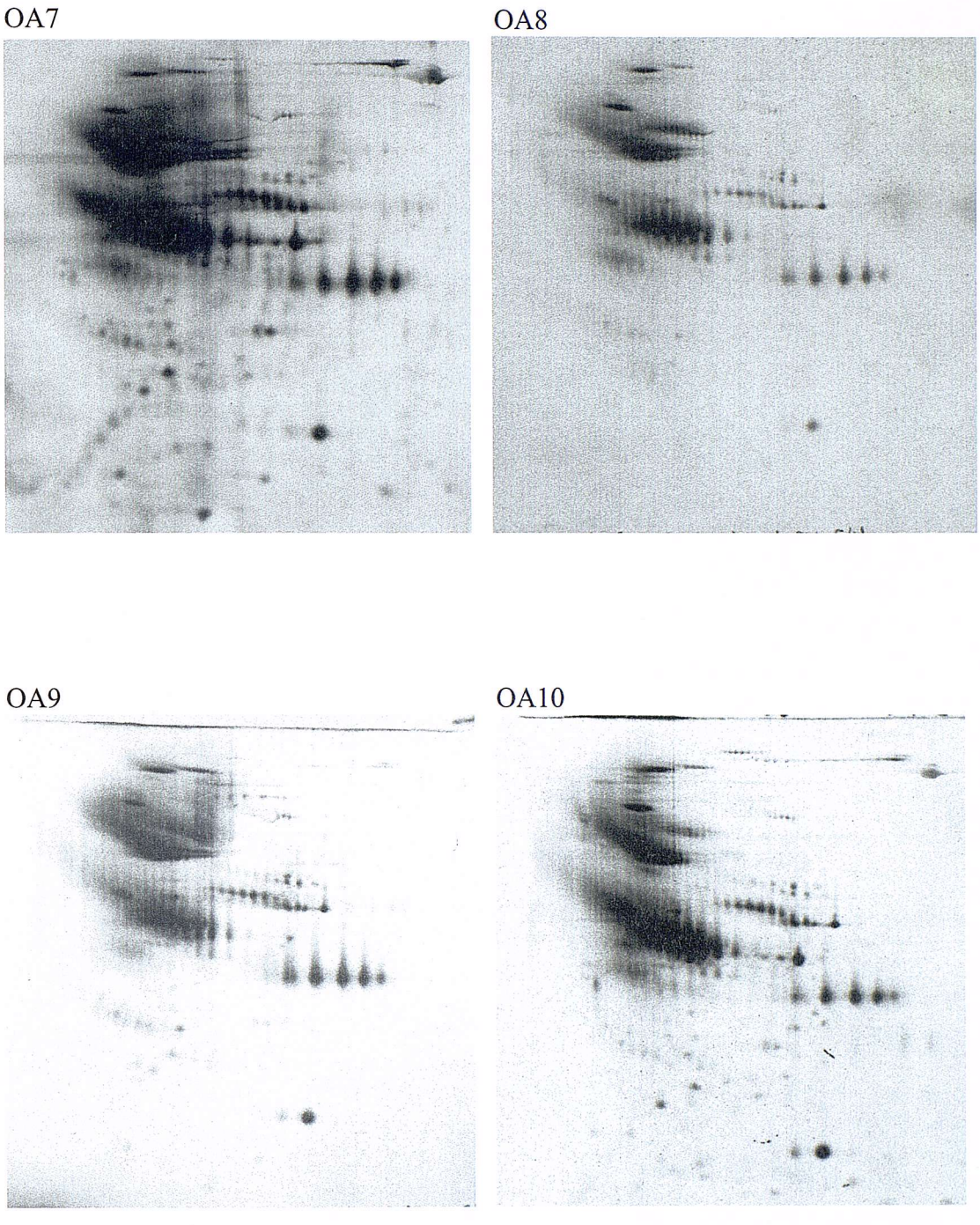


Fig 5.5. Autoradiographs of OA human explant media samples OA1-OA10.

Table 5.2 **Proteins identified from human OA cartilage explant medium (OA1) by 2D-electrophoresis and MALDI and ESI MS.**
a. by spot number. b. in alphabetical order

Table 5.2a.

Spot	Protein
A1	Inhibin beta A
A2	Cytokine-like protein C17
A3	Cytokine-like protein C17
A4	Tetranectin
A5	Tetranectin
A6	Tetranectin
A7	Tetranectin
A8	Tetranectin
A9	Metalloproteinase inhibitor 2 (TIMP-2)
A10	Metalloproteinase inhibitor 2 (TIMP-2)
A11	Metalloproteinase inhibitor 1 (TIMP-1)
A12	Metalloproteinase inhibitor 1 (TIMP-1)
B1	Metalloproteinase inhibitor 1 (TIMP-1)
B2	Metalloproteinase inhibitor 1 (TIMP-1)
B3	Osteoinductive factor
B4	Clusterin
B5	Clusterin
B6	Osteoinductive factor Clusterin
B7	Osteoinductive factor
B8	Osteoinductive factor Clusterin
B9	Osteoinductive factor
B10	Osteoinductive factor Clusterin
B11	Osteoinductive factor
B12	Osteoinductive factor Osteonectin/SPARC
C1	Osteonectin/SPARC
C2	Vimentin Osteonectin/SPARC
C3	Vimentin
C4	Vimentin
C5	Vimentin
C6	Vimentin
C7	Osteoinductive factor
C8	Osteoinductive factor
C9	Clusterin
C10	Clusterin Cathepsin L
C11	Lamin
C12	Clusterin
D1	Clusterin
D2	Inhibin beta A
D3	Inhibin beta A
D4	Inhibin beta A
D5	Inhibin beta A
D6	Inhibin beta A
D7	Inhibin beta A
D8	Inhibin beta A
D9	YKL-39/chitinase-3 like protein 2
D10	YKL-40/gp-39/chitinase-3 like protein 1
D11	YKL-40/gp-39/ chitinase-3 like protein 1 Fructose-bisphosphate aldolase
D12	Alpha-1-antitrypsin
E1	YKL-40/gp-39/ chitinase-3 like protein 1
E2	YKL-40/gp-39/ chitinase-3 like protein 1
E3	Matrix metalloproteinase-3 (MMP-3)
E4	Matrix metalloproteinase-3 (MMP-3)
E5	Matrix metalloproteinase-3 (MMP-3)
E6	Matrix metalloproteinase-2 (MMP-2)
E7	ASPIC Lumican
E8	Lumican
E9	Collagen alpha 1 type VI
E10	Complement factor B
E11	Complement factor B
E12	Complement factor B
F1	Complement factor B
F2	Complement factor B
F3	Precursor polypeptide (AA-14 to 747) Kinesin-like protein
F4	Putative DNA/chromatin binding motif
F5	Cartilage intermediate layer protein (CILP)
F6	Cartilage intermediate layer protein (CILP)
F7	Cartilage intermediate layer protein (CILP)
F8	Cartilage intermediate layer protein (CILP)
F9	Cartilage intermediate layer protein (CILP)
F10	Cartilage intermediate layer protein (CILP)
F11	Cartilage intermediate layer protein (CILP)

F12	Golgin-160
G1	Golgin-160
G2	L-lactate dehydrogenase
G3	L-lactate dehydrogenase
G4	Collagen alpha 1 type II C-propetide
G5	Collagen alpha 1type II C-propeptide
G6	Glyceraldehyde 3-phosphate dehydrogenase
G7	Glyceraldehyde 3-phosphate dehydrogenase
G8	Glyceraldehyde 3-phosphate dehydrogenase
G9	Glyceraldehyde 3-phosphate dehydrogenase
G10	Glyceraldehyde 3-phosphate dehydrogenase
G11	Procollagen C-proteinase enhancer protein P
G12	Procollagen C-proteinase enhancer protein P
H1	Procoallgen C-proteinase enhancer protein P
H2	Procollagen C-proteinase enhancer protein P
H3	Procollagen C-proteinase enhancer protein P
H4	Procollagen C-proteinase enhancer protein P
H5	Extracellular superoxide dismutase [Cu-Zn]
H6	Extracellular superoxide dismutase [Cu-Zn]
H7	Extracellular superoxide dismutase [Cu-Zn]
H8	Carbonic anhydrase
H9	Carbonic anhydrase
H10	Complement factor D
H11	Complement factor D
H12	Complement factor D
2A1	Biliverdin reductase B
2A2	Phosphatidylethanolamine-binding protein
2A3	Serum amyloid P
2A4	Cartilage oligomeric matrix protein (COMP)
2A5	Cartilage oligomeric matrix protein (COMP)
2A6	α-1-microglobulin-bikunin Cartilage intermediate layer protein (CILP) Cartilage oligomeric matrix protein (COMP)
2A7	Cartilage oligomeric matrix protein (COMP)
2A8	Peroxiredoxin 2
2A9	Peroxiredoxin 2
2A10	Destrin (Actin-depolymerizing factor)
2A11	Transthyretin (prealbumin)
2A12	Peptidyl-prolyl cis-trans isomerase B
2B1	Peptidyl-prolyl cis-trans isomerase
2B2	Chain A, zinc dependent dimers Type XVIII collagen
2B3	Haemoglobin alpha chain
2B4	Haemoglobin alpha chain
2B5	Haemoglobin alpha chain

2B6	Lysozyme C
2B7	Profilin 1
2B8	Haemoglobin delta chain
2B9	Haemoglobin beta chain
2B10	Haemoglobin beta chain
2B11	Haemoglobin beta chain
2B12	Haemoglobin delta chain
2C1	Haptoglobin-1
2C2	Retinoic acid receptor responder protein 2
2C3	Retinoic acid receptor responder protein 2
2C4	Thrombospondin 4 N terminal domain
2C5	Ribonuclease pancreatic precursor
2C6	Glutathione S-transferase
2C7	Prolargin
2C8	Prolargin
2C9	Phosphoglycerate mutase
2C10	Ig kappa chain region
2C11	Ig kappa chain region
2C12	Chondroadherin
2D1	Chondroadherin
2D2	Annexin II
2D3	Annexin II
2D4	Complement C4
2D5	Hypothetical 187.1 kDa protein in OGG1-CNA2
2D6	Ribonuclease 4
2D7	Cofilin
2D8	C-type lectin
2D9	Superoxide dismutase [Cu-Zn]
2D10	Apolipoprotein A-1
2D11	Apolipoprotein A-1
2D12	Triosephosphate isomerase
2E1	Triosephosphate isomerase
2E2	Complement C1q subcomponent, B chain
2E3	Collagen alpha 1 (XI) chain
2E4	Annexin V
2E5	Annexin V
2E6	Fibrinogen beta chain
2E7	Pigment epithelium-derived factor
2E8	Pigment epithelium-derived factor
2E9	Pigment epithelium-derived factor
2E10	Pigment epithelium-derived factor
2E11	Pigment epithelium-derived factor
2E12	Pigment epithelium-derived factor
2F1	Phosphoglycerate kinase
2F2	Serine protease HTRA1

2F3	Ig gamma-2 chain C region
2F4	Complement C3 β-chain
2F5	Serotransferrin
2F6	Serotransferrin
2F7	Serotransferrin
2F8	Serotransferrin
2F9	Fibromodulin
2F10	Fibromodulin
2F11	Alpha-1B glycoprotein
2F12	Hemopexin
2G1	Annexin I
2G2	Related to glycerol-3-phosphate dehydrogenase
2G3	H factor (complement)-like 1
2G4	Actin, aortic smooth muscle (α-actin 2)
2G5	Procollagen C-endopeptidase enhancer
2G6	Hypothetical 72.2 kDa protein
2G7	Hypothetical 72.2 kDa protein
2G8	Hypothetical 72.2 kDa protein
2G9	Ig gamma-4 chain C region
2G10	Beta-2-glycoprotein I
2G11	Ig gamma-1 chain C region
2G12	Polynucleotide nucleotidyltransferase

Table 5.2b

Spot	Protein
2A6	α-1-microglobulin-bikunin
2G4	Actin, aortic smooth muscle (α-actin 2)
D12	Alpha-1-antitrypsin
2F11	Alpha-1B glycoprotein
2G1	Annexin I
2D2	Annexin II
2D3	Annexin II
2E4	Annexin V
2E5	Annexin V
2D10	Apolipoprotein A-1
2D11	Apolipoprotein A-1
E7	ASPIC
2G10	Beta-2-glycoprotein I
2A1	Biliverdin reductase B
H8	Carbonic anhydrase
H9	Carbonic anhydrase
F5	Cartilage intermediate layer protein (CILP)
F6	Cartilage intermediate layer protein (CILP)
F7	Cartilage intermediate layer protein (CILP)
F8	Cartilage intermediate layer protein (CILP)
F9	Cartilage intermediate layer protein (CILP)
F10	Cartilage intermediate layer protein (CILP)
F11	Cartilage intermediate layer protein (CILP)
2A6	Cartilage intermediate layer protein (CILP)
2A4	Cartilage oligomeric matrix protein (COMP)
2A5	Cartilage oligomeric matrix protein (COMP)
2A6	Cartilage oligomeric matrix protein (COMP)
2A7	Cartilage oligomeric matrix protein (COMP)
C10	Cathepsin L
2B2	Chain A, zinc dependent dimers
2C12	Chondroadherin
2D1	Chondroadherin
B4	Clusterin
B5	Clusterin
B6	Clusterin
B8	Clusterin
B10	Clusterin
C9	Clusterin
C10	Clusterin
C12	Clusterin
D1	Clusterin
2D7	Cofilin
2E3	Collagen alpha 1 (XI) chain
G4	Collagen alpha 1 type II C-propetide
E9	Collagen alpha 1 type VI
G5	Collagen alpha 1type II C-propeptide
2E2	Complement C1q subcomponent, B chain
2F4	Complement C3 β-chain
2D4	Complement C4
E10	Complement factor B
E11	Complement factor B
E12	Complement factor B
F1	Complement factor B
F2	Complement factor B
H10	Complement factor D

H11	Complement factor D
H12	Complement factor D
2D8	C-type lectin
A2	Cytokine-like protein C17
A3	Cytokine-like protein C17
2A10	Destrin (Actin-depolymerizing factor)
H6	Extracellular superoxide dismutase [Cu-Zn]
H7	Extracellular superoxide dismutase [Cu-Zn]
H5	Extracellular superoxide dismutase [Cu-Zn]
2E6	Fibrinogen beta chain
2F9	Fibromodulin
2F10	Fibromodulin
D11	Fructose-bisphosphate aldolase
2C6	Glutathione S-transferase
G6	Glyceraldehyde 3-phosphate dehydrogenase
G7	Glyceraldehyde 3-phosphate dehydrogenase
G8	Glyceraldehyde 3-phosphate dehydrogenase
G9	Glyceraldehyde 3-phosphate dehydrogenase
G10	Glyceraldehyde 3-phosphate dehydrogenase
F12	Golgin-160
G1	Golgin-160
2G3	H factor (complement)-like 1
2B3	Haemoglobin alpha chain
2B4	Haemoglobin alpha chain
2B5	Haemoglobin alpha chain
2B9	Haemoglobin beta chain
2B10	Haemoglobin beta chain
2B11	Haemoglobin beta chain
2B8	Haemoglobin delta chain
2B12	Haemoglobin delta chain
2C1	Haptoglobin-1
2F12	Hemopexin
2D5	Hypothetical 187.1 kDa protein in OGG1-CNA2
2G6	Hypothetical 72.2 kDa protein
2G7	Hypothetical 72.2 kDa protein
2G8	Hypothetical 72.2 kDa protein
2G11	Ig gamma-1 chain C region
2F3	Ig gamma-2 chain C region
2G9	Ig gamma-4 chain C region
2C10	Ig kappa chain region
2C11	Ig kappa chain region
A1	Inhibin beta A
D2	Inhibin beta A
D3	Inhibin beta A
D4	Inhibin beta A
D5	Inhibin beta A
D6	Inhibin beta A
D7	Inhibin beta A
D8	Inhibin beta A
F3	Kinesin-like protein
C11	Lamin
G2	L-lactate dehydrogenase
G3	L-lactate dehydrogenase
E7	Lumican
E8	Lumican
2B6	Lysozyme C
E6	Matrix metalloproteinase-2 (MMP-2)
E3	Matrix metalloproteinase-3 (MMP-3)
E4	Matrix metalloproteinase-3 (MMP-3)
E5	Matrix metalloproteinase-3 (MMP-3)
A11	Metalloproteinase inhibitor 1 (TIMP-1)
A12	Metalloproteinase inhibitor 1 (TIMP-1)
B1	Metalloproteinase inhibitor 1 (TIMP-1)
B2	Metalloproteinase inhibitor 1 (TIMP-1)
A9	Metalloproteinase inhibitor 2 (TIMP-2)
A10	Metalloproteinase inhibitor 2 (TIMP-2)
B3	Osteoinductive factor
B6	Osteoinductive factor
B7	Osteoinductive factor
B8	Osteoinductive factor
B9	Osteoinductive factor
B10	Osteoinductive factor
B11	Osteoinductive factor
B12	Osteoinductive factor
C7	Osteoinductive factor
C8	Osteoinductive factor
B12	Osteonectin/SPARC
C1	Osteonectin/SPARC
C2	Osteonectin/SPARC
2B1	Peptidyl-prolyl cis-trans isomerase
2A12	Peptidyl-prolyl cis-trans isomerase B
2A8	Peroxiredoxin 2
2A9	Peroxiredoxin 2
2A2	Phosphatidylethanolamine-binding protein
2F1	Phosphoglycerate kinase
2C9	Phosphoglycerate mutase
2E7	Pigment epithelium-derived factor
2E8	Pigment epithelium-derived factor
2E9	Pigment epithelium-derived factor
2E10	Pigment epithelium-derived factor

2E11	Pigment epithelium-derived factor
2E12	Pigment epithelium-derived factor
2G12	Polynucleotide nucleotidyltransferase
F3	Precursor polypeptide (AA-14 to 747)
H1	Procoallgen C-proteinase enhancer protein P
2G5	Procollagen C-endopeptidase enhancer
G11	Procollagen C-proteinase enhancer protein P
G12	Procollagen C-proteinase enhancer protein P
H2	Procollagen C-proteinase enhancer protein P
H3	Procollagen C-proteinase enhancer protein P
H4	Procollagen C-proteinase enhancer protein P
2B7	Profilin 1
2C7	Prolargin
2C8	Prolargin
F4	Putative DNA/chromatin binding motif
2G2	Related to glycerol-3-phosphate dehydrogenase
2C2	Retinoic acid receptor responder protein 2
2C3	Retinoic acid receptor responder protein 2
2D6	Ribonuclease 4
2C5	Ribonuclease pancreatic precursor
2F2	Serine protease HTRA1
2F5	Serotransferrin
2F6	Serotransferrin
2F7	Serotransferrin
2F8	Serotransferrin
2A3	Serum amyloid P
2D9	Superoxide dismutase [Cu-Zn]
A4	Tetranectin
A5	Tetranectin
A6	Tetranectin
A7	Tetranectin
A8	Tetranectin
2C4	Thrombospondin 4 N terminal domain
2A11	Transthyretin (prealbumin)
2D12	Triosephosphate isomerase
2E1	Triosephosphate isomerase
2B2	Type XVIII collagen
C2	Vimentin
C3	Vimentin
C4	Vimentin
C5	Vimentin
C6	Vimentin
D9	YKL-39/chitinase-3 like protein 2
D11	YKL-40/gp-39/ chitinase-3 like protein 1
E1	YKL-40/gp-39/ chitinase-3 like protein 1
E2	YKL-40/gp-39/ chitinase-3 like protein 1
D10	YKL-40/gp-39/chitinase-3 like protein 1

5.3 Inhibin βA is upregulated in OA explant medium

A row of diagonal spots with a molecular weight of about 45 kDa, together with a protein of 14 kDa were upregulated in OA explant medium samples compared to normal (see fig 5.1 and fig. 5.3 and all gels in 5.4 and 5.5). MS analysis identified the upper protein spots as pro-inhibin βA and the 14 kDa protein as the mature inhibin βA/activin A.

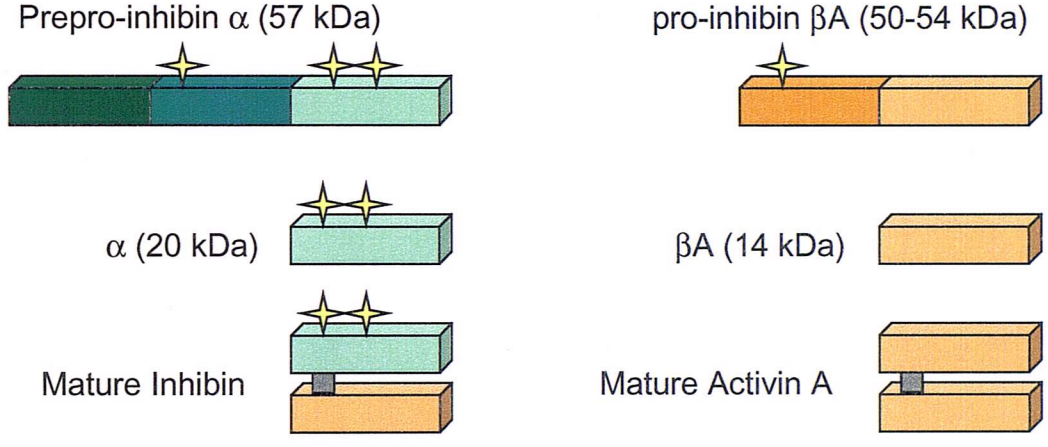


Fig 5.6. Subunit structure and composition of inhibin and activin. Both protein subunits are synthesised as precursors. Prepro-α is cleaved to form mature α-subunit and pro-βA is cleaved to form mature β-subunit. Inhibin is formed by heterodimerisation of two α-subunits and activin A is formed by assembly of one α-subunit and one β-subunit. The dimers are disulphide-linked. Both precursors are glycosylated, whereas only the mature α-subunit is (indicated by a 'star'). The size of each subunit is given after the name.

Activin and inhibin belong to the TGFβ superfamily (reviewed in Bernard et al., 2001). They were originally found in ovarian follicular fluid. Activin stimulated, but inhibin inhibited, follicle-stimulating hormone (FSH) release from pituitary cells. Both are disulphide-linked dimers of subunits processed from larger precursors. The dimers are made up of one or two subunit types, an inhibin α-chain and an inhibin β-chain (fig 5.6). Three β-chain genes have been identified in human, called β_A, β_B and β_C. Activin consists only of β-chains and the nomenclature reflects the type of β-subunits as follows: activin A (β_A-β_A), activin B (β_B-β_B) and activin AB (β_A-β_B) etc. Inhibin is made up of one α-chain and one β-chain. The inhibin nomenclature reflects the type of β-subunit in the heterodimer: inhibin A (α-βA), inhibin B (α-βB) etc. It was likely that the protein upregulated in OA chondrocytes was activin A, since no inhibin α-chain or other types of β-chains were detected.

The amounts of pro-inhibin βA and inhibin βA/activin A were compared between samples by measuring the area and intensity of the protein spots on the autoradiograph and expressing the values as unit protein per unit of the central spot of TIMP-1 (fig 5.7 and 5.8). TIMP-1 was used for normalisation between autoradiographs. Only samples from load bearing joints were compared (hip, knee, ankle).

Pro-inhibin βA was detected in all OA explant medium samples (0.5-3.2 unit protein /unit TIMP-1) and the mature activin A was detected (0.1-2.0 unit protein/unit TIMP-1) in all but the OA4 sample (0.03 unit protein/unit TIMP-1). Inhibin βA (pro and mature forms) was detected in three of the non-OA explant medium samples (N10-12, see table 5.1). Interestingly, these samples were derived from humeral head cartilage of a patient with adjacent metastasis of a breast carcinoma (N10), from ankle joint cartilage from a limb amputated for osteomyelitis (N11), and from cartilage of a humeral head excised because of avascular necrosis (N12). In all other normal or non-OA samples, activin was undetectable or present at less than 0.06 unit protein/unit TIMP-1.

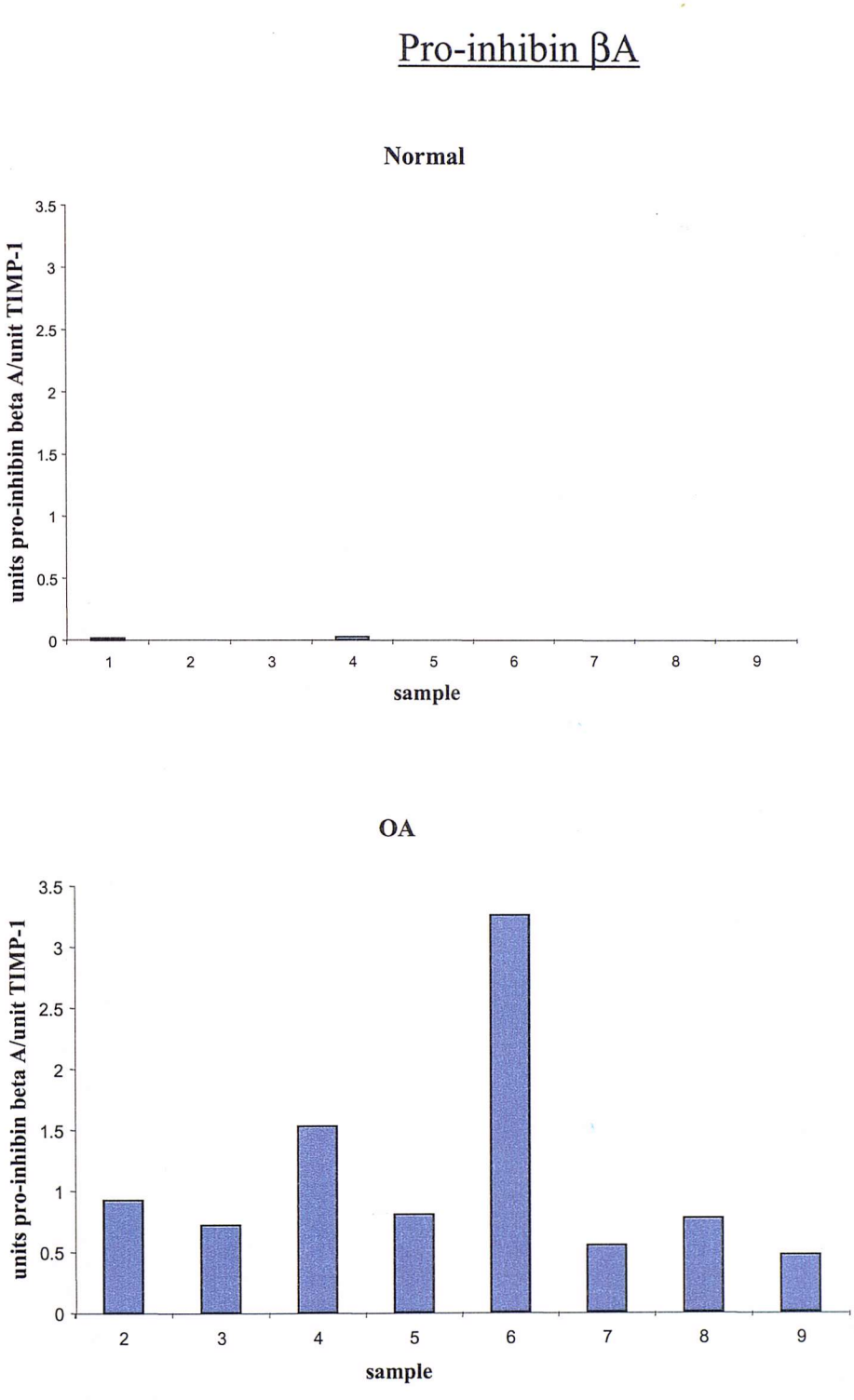


Fig 5.7. Comparison of pro-inhibin βA in normal and OA cartilage explant medium samples. Nine normal samples were compared with eight OA samples. The central TIMP-1 spot on the autoradiographs were used for normalization of absorbance intensity of proteins. The data was analysed with Phoretix 2D software version 6.01 (Nonlinear Dynamic Ltd, UK).

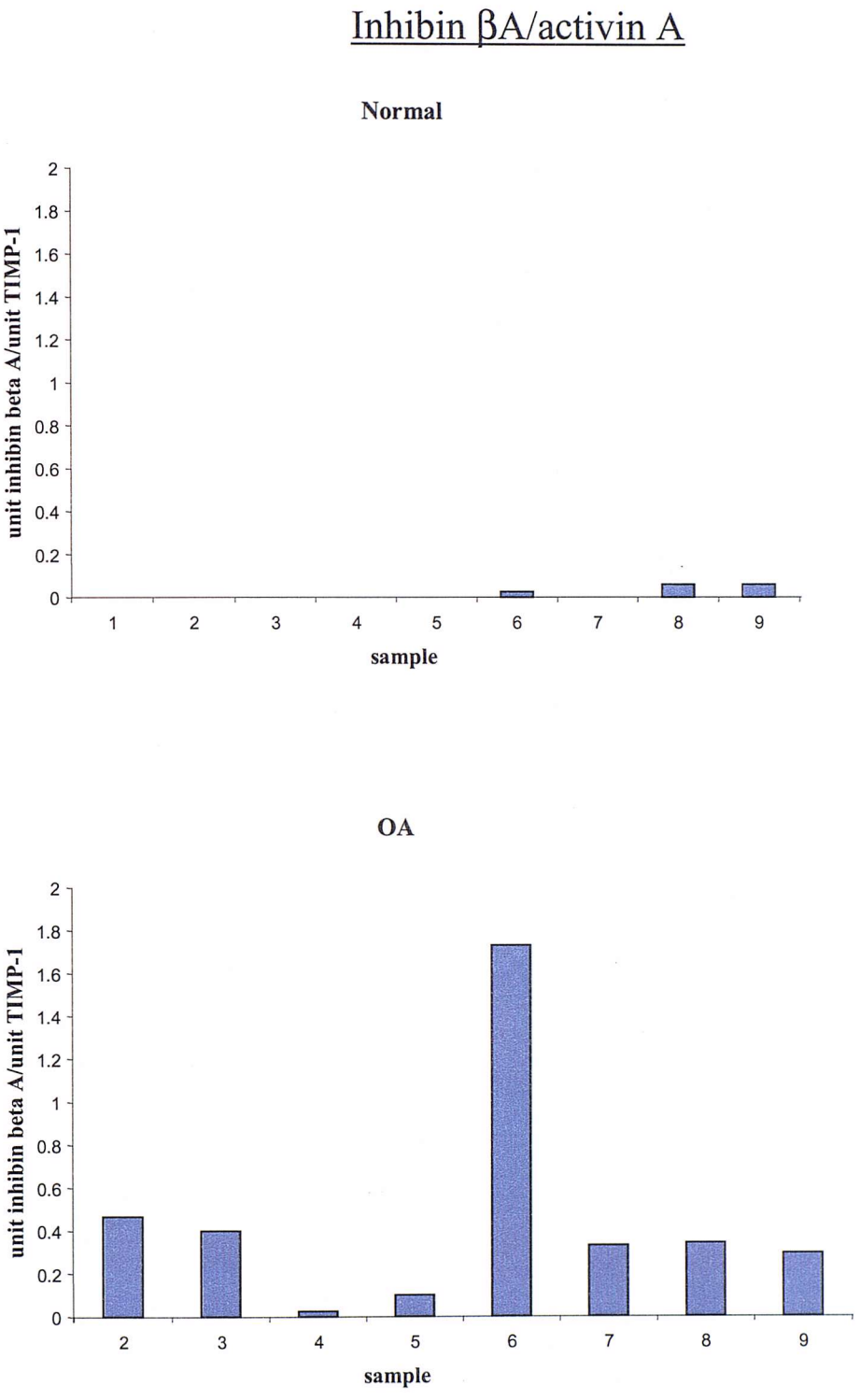


Fig 5.8. Comparison of Inhibin βA/activin A in normal and OA cartilage explant medium samples. Nine normal samples were compared with eight OA samples. The central TIMP-1on the autoradiographs were used for normalization of absorbance intensity of proteins. The data was analysed with Phoretix 2D software version 6.01 (Nonlinear Dynamic Ltd, UK).

5.3.1 No inhibin βA detected in porcine explant medium

The porcine homologues of pro-inhibin βA and inhibin βA/activin A were not detected in porcine explant media. Generally the porcine explants were rested for three days before metabolic labelling. This was done to make sure any gene expression induced by dissection would return to basal levels prior to cytokine stimulation. It was possible that inhibin βA could have been produced transiently and washed away during the resting period and therefore were not detected. If porcine chondrocytes produced inhibin βA, it would be a useful model for studies of the role of inhibin βA in cartilage.

An experiment was set up to study whether inhibin βA could be detected in medium of freshly dissected porcine explants. Porcine cartilage was explanted and treated with medium containing radiolabelled amino acids, as described for human cartilage. After conditioning, the medium was treated with 3 mg CPC/mg GAG for 30 min at room temperature, dialysed against water at 4 °C and lyophilized. The sample was analysed by 2D-electrophoresis and the gel stained with silver. The metabolically labelled protein pattern was similar to that obtained with explants after three days culture (fig 5.9 and compare with fig 4.12c). The major stainable and labelled protein spots were the C-propeptide and α-chain of collagen type II, osteonectin and TIMP-1. No candidate spots likely to be inhibin βA were detected. Since the pig and human orthologues are 88 % identical and their βA-chains have identical pI and molecular weight, the pig βA-chain would be detected if present under the conditions used. It was therefore concluded that porcine chondrocytes in explants do not express inhibin βA in detectable amounts, and the induction found in human OA cartilage could be disease related.

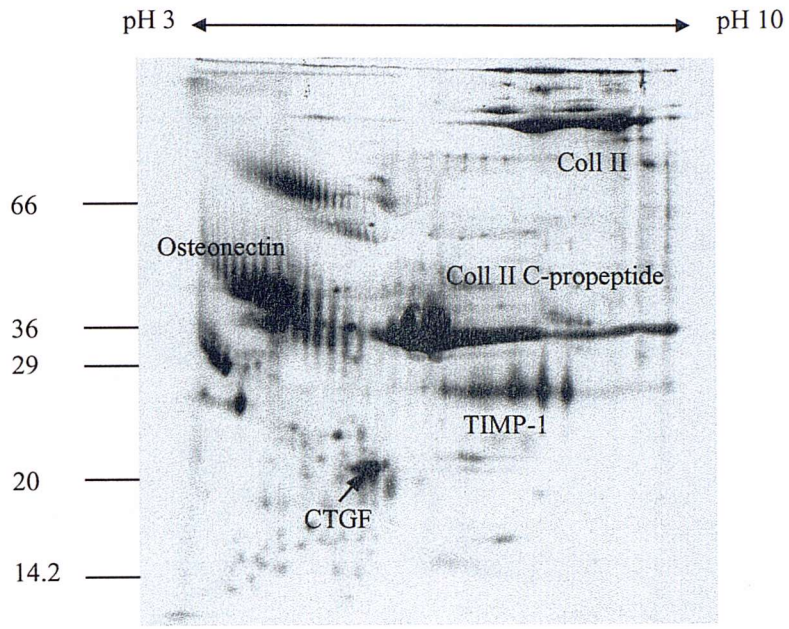


Fig 5.9. Autoradiograph of SDS-PAGE of secreted proteins from porcine explant medium pulsed on day 1. 6 g of articular porcine cartilage was removed and washed in serum free DMEM overnight. The following day, the cartilage was pulsed with [35S]methionine/cysteine (200 µCi/ml) for 5h. Explant medium was removed and treated with 3 mg CPC/mg GAG for 30 min at room temperature. The supernatant was removed and dialysed into water at 4°C and lyophilized. Sample was isoelectric focused on a pH 3-10 gradient. Focused proteins were electrophoresed by SDS-PAGE on a 12 % gel. The gel was stained with silver, dried and exposed to film for 5 days. The gel was rehydrated and protein spots excised, trypsinized and identified by MS.

5.4 Synthesis of collagen type II is upregulated in OA explants

Visual inspection suggested that expression of both the collagen II α-chain and the collagen II C-propeptide were upregulated in OA explant medium compared to the normal samples (fig 5.3). Because the putative α-chain often focused poorly, the labelled C-propeptide was used as a measure of the amount of type II collagen synthesised. As in the porcine medium, the C-propeptide was observed as a major spot, accompanied by one or two minor more acidic ones (see fig 5.3a). Often only the major spot was detectable on the autoradiographs (e.g. fig 5.1). This was measured by densitometric scanning and the measurement normalised to the density of the central TIMP-1 protein spot as for inhibin. Only samples from load bearing cartilage was compared. The C-propeptide was detected (0.05-2.3 units/unit TIMP-1) in all OA explant medium samples, though in OA8, the signal was very weak (0.009 unit protein/unit TIMP-1) (fig 5.10). In normal explant medium samples the C-propeptide was either undetectable or present at less than 0.02 unit protein/unit TIMP-1). No synthesis of C-propeptide was observed in the non-OA explant medium samples (N10-N12).

5.4.1 New synthesis of collagen type II in very young cartilage

One normal human explant medium sample contained a relatively large amount of labelled C-propeptide (1.3 units/unit TIMP-1). This sample had come from Belgium and was from a femoral condyle of a 7-week-old infant (N13, see table 5.1 and fig 5.4). It is likely that collagen type II is synthesised in larger amounts in growing individuals than in adults. The porcine cartilage used in the experiments described in chapter 3 and 4 was from 6 month old animals and made significant quantities of collagen type II. Pro-inhibin βA and inhibin βA levels in the human infant sample were low (by visual inspection of the autoradiograph).

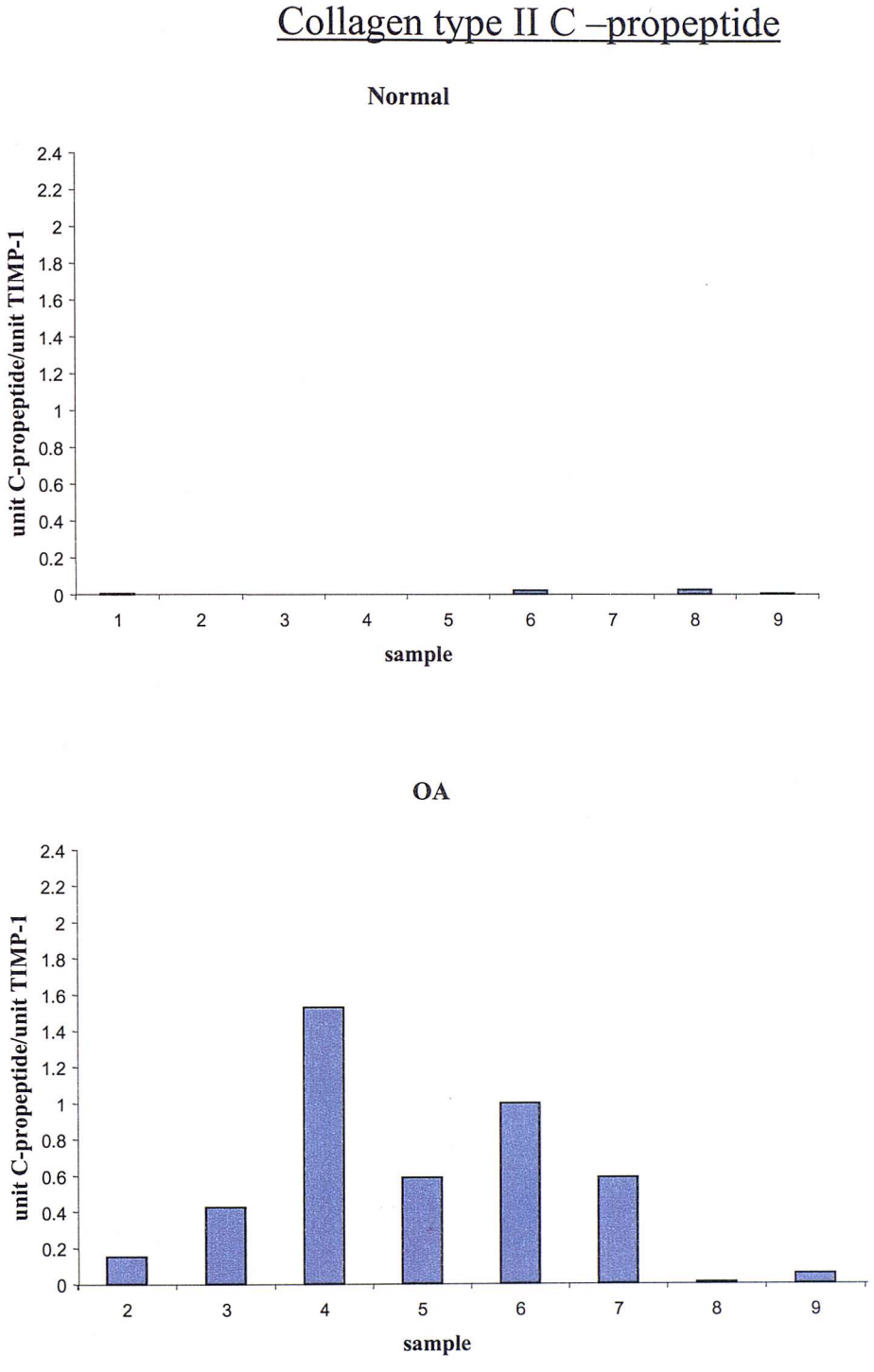


Fig 5.10. Comparison of C-propeptide of collagen type II in normal and OA cartilage explant medium. Nine normal samples were compared with eight OA samples. The central TIMP-1 on the autoradiographs were used for normalization of absorbance intensity of proteins. The data was analysed with Phoretix 2D software version 6.01 (Nonlinear Dynamic Ltd, UK).

5.5 Possible regulation of YKL-40/gp-39 in OA explant medium

It has been suggested that YKL-40/gp-39 plays a role in remodelling and degradation of cartilage (Hakala et al., 1993; Verheijden et al., 1997). YKL-40 was detected as a set of 3 or 4 labelled protein spots in 9/13 (70 %) of the normal, but only in 3/10 (30 %) of the OA explant medium samples (table 5.3). It was not detected in the "normal" explants from the samples diagnosed as osteomyelitis and avascular necrosis (N11 and N12), however it was present in the normal sample obtained from a patient with metastatic breast carcinoma (N10). More samples need to be analysed to determine whether production of YKL-40 is down regulated in OA cartilage.

Table 5.3 The presence of YKL-40 in explant medium samples.

Sample	Diagnosis	YKL-40	Sample	Diagnosis	YKL-40
N1	normal	+	OA1	OA	+
N2	normal	+	OA2	OA	+
N3	normal	+	OA3	OA	-
N4	normal	+	OA4	OA	-
N5	normal	-	OA5	OA	+
N6	normal	+	OA6	OA	-
N7	normal	-	OA7	OA	-
N8	normal	+	OA8	OA	-
N9	normal	+	OA9	OA	-
N10	normal[1]	+	OA10	OA	-
N11	normal[2]	-			
N12	normal[3]	-			
N13	normal	+			

[1] patient diagnosed with metastatic breast cancer

[2] amputation due to osteomyelitis

[3] excision following avascular necrosis

5.6 Discussion

2D-electrophoresis of metabolically labelled medium samples of normal and OA explant medium was performed. Approximately 250 spots were resolved by 2D-electrophoresis of which about 50-100 protein spots were labelled. So far, 18 labelled proteins have been identified by MS (fig 5.2 and 5.3). Of the proteins identified, many have been reported to be highly expressed in cartilage. These include collagen type II, COMP, clusterin, lumican, CTGF and others. Several un-labelled cartilage proteins were also found like fibromodulin and CILP. Many of the proteins identified were present in the gels as multiple spots. These were likely to be differently post-translationally modified forms that give rise to altered molecular weights and isoelectric points. This was unsurprising in the case of glycosylated proteins such as lumican and clusterin.

In total 179 excised spots were identified, corresponding to 92 distinct gene products from the OA1 sample (table 5.2. and Appendix 2).

5.7 Proteins reported as induced in OA cartilage, not detected by 2D-electrophoresis and MS

Inevitably the proteomic analysis excluded aggrecan because it was removed by CPC precipitation. Proteins interacting with aggrecan (e.g. link protein) were also likely to be lost to the analysis, as were possibly other proteoglycans. Large proteins (e.g. fibronectin) were also likely to be poorly represented in the 2D gels. Proteins larger than 200 kDa are often not observed on 2D-gels, presumably due to insufficient transfer between the first and second dimension.

Aggrecan cleavage products generated by aggrecanase have been identified in cultures undergoing cartilage matrix degradation, and in synovial fluid of OA patients (Lohmander et al., 1999; Sandy et al., 1991). No aggrecanase was detected in OA human cartilage explant medium samples by 2D-electrophoresis. It is not known if aggrecanase would focus on a 2D-gel and it is likely that the levels of aggrecanase in

the samples would be below detection levels. Moreover, aggrecanase could be lost in the CPC precipitation of aggrecan.

It has been reported that fibronectin (FN) and fibronectin fragments (FN-fs) are elevated in human OA cartilage and synovial fluid (Homandberg, 1999). In this study no FN or FN-fs were detected in OA explant medium by 2D-electrophoresis, though full-length FN was detected in normal and OA cartilage by 1D-electrophoresis (*chapter 3*). The level of FN-fs were suggested to be as high a ~570 μg/ml in synovial fluid from OA patients (Homandberg, 1999) however the Fn-fs might not necessarily all be derived from the chondrocytes.

The product of TNF-stimulated gene-6 (TSG-6) is a hyaluronan-binding protein, likely to play a role in extracellular matrix remodelling (Lee et al., 1992). TSG-6 has been found in synovial fluid from patients with arthritis (Wisniewski et al., 1993). Immunolocalisation of TSG-6 in normal and OA cartilage showed that the protein was present in OA tissue only (Bayliss et al., 2001b). TSG-6 was not detected by 2D-electrophoresis.

Osteopontin, a highly phosphorylated and sulphated glycoprotein (35-70 kDa) generally expressed by hypertrophic chondrocytes, is reportedly induced in OA chondrocytes (Pullig et al., 2000). A qualitative analysis indicated that the osteopontin protein and mRNA expression increased with the severity of the OA lesions. This protein has not been detected with our method or by sequencing of OA cartilage cDNA libraries (Kumar et al., 2001). It is possible that osteopontin is lost in the CPC precipitation procedure due to its polyanionic structure.

An enhanced expression of collagen type X has been reported to be a late stage characteristic of OA (Pullig et al., 1999). Type X collagen was not identified on any of our gels.

5.8 Investigation of proteoglycans and associated proteins in explant medium

When explant medium samples were prepared for 2D-electrophoresis, the proteoglycan and any proteins binding strongly to them were removed by CPC precipitation. To identify the proteoglycans, the pellet could be digested with

chondroitinase ABC lyase and the samples western blotted with specific antibodies to the various core proteins. Further analysis of interesting proteins could be made directly on the pellet run in 1D or 2D. So far link protein has been identified from a 1D-gel (data not shown).

5.9 Activin A is induced in OA cartilage

Pro-inhibin βA and inhibin βA were upregulated in OA explant medium samples compared to controls. The absence of other types of inhibin chains suggested that the upregulated precursor protein was generating activin A. It has not previously been described to be upregulated in OA cartilage. No other TGFβ-family members were detected although to find TGFβ or BMPs might have been expected.

5.9.1 Activin A signalling

Activin A is related to TGFβ (fig 5.11) and the high degree of conservation is mirrored in the preservation of the receptors and intracellular signalling components. The receptors for activin are transmembrane serine/threonine kinases like their TGFβ counterparts. Similarly to TGFβ receptors, there is a type II ligand-binding receptor (ActRII) and a type I signalling receptor (ActRI). Activin binds to ActRII, which recruits the ActRI. Activation of the ActRI leads to phosphorylation of transcription factors known as signalling mothers against decapentaplegic homolog 2 and 3 (Smad 2 and Smad 3). They transduce the signal from the cytoplasm to the nucleus through an interaction with common Smad, Smad 4. Both activin and TGFβ signal through Smad 2 and Smad 3 (Pangas and Woodruff, 2000).

In many biological systems, activin signalling is antagonised by its related heterodimer, inhibin, as mentioned previously in the case of FSH release. Inhibin binds to the ActRII with 10-fold less affinity than activin, thus antagonism occurs when inhibin is present at higher concentrations than activin (Mathews and Vale, 1991). The ligands also have opposing roles during chondrogenesis by chick limb bud mesodermal cells (Chen et al., 1993), and in the proliferation of T cells (Hedger et al.,

1989). The effect of activin is not opposed by inhibin in mesodermal induction (Thomsen et al., 1990), neuronal cell survival (Schubert et al., 1990) and in some developmental pathways (Merino et al., 1999).

The relatively weak affinity of inhibin for ActRII fails to explain its high potency as an activin antagonist in some systems. Therefore, it was suggested that there might be a distinct high-affinity inhibin receptor. The TGFβ type III receptor, betaglycan, is a candidate inhibin receptor (Lewis et al., 2000). It binds inhibin with high affinity. When betaglycan and ActRII are present together on the same cell, betaglycan increases the ability of inhibin to bind to ActRII. This presumably occurs via formation of a ternary complex. Therefore inhibin sequesters ActRII and so prevents ActRI and RII forming a signalling complex. A 120 kDa membrane-anchored proteoglycan purified from bovine pituitary membranes may be a physiological receptor for inhibin (Chong et al., 2000). This protein bound strongly to inhibin and was unrelated to the other receptors proteins in the family. It was later found to be actually 142 kDa in size and was renamed inhibin binding protein (InhBP) (Bernard et al., 2001).

Follistatin is another factor that can inhibit activin signalling. This glycoprotein binds both activin and inhibin through their beta-chains. When complexed with activin or inhibin, follistatin neutralises all ligand activity (Nakamura et al., 1990). Follistatin-deficient mice have more widespread defects then activin-deficient mice, indicating that follistatin may modulate the actions of molecules other than activin, and possibly other members of the TGFβ-family (Matzuk et al., 1995b). Follistatin may function in vivo to present activins to their receptors.

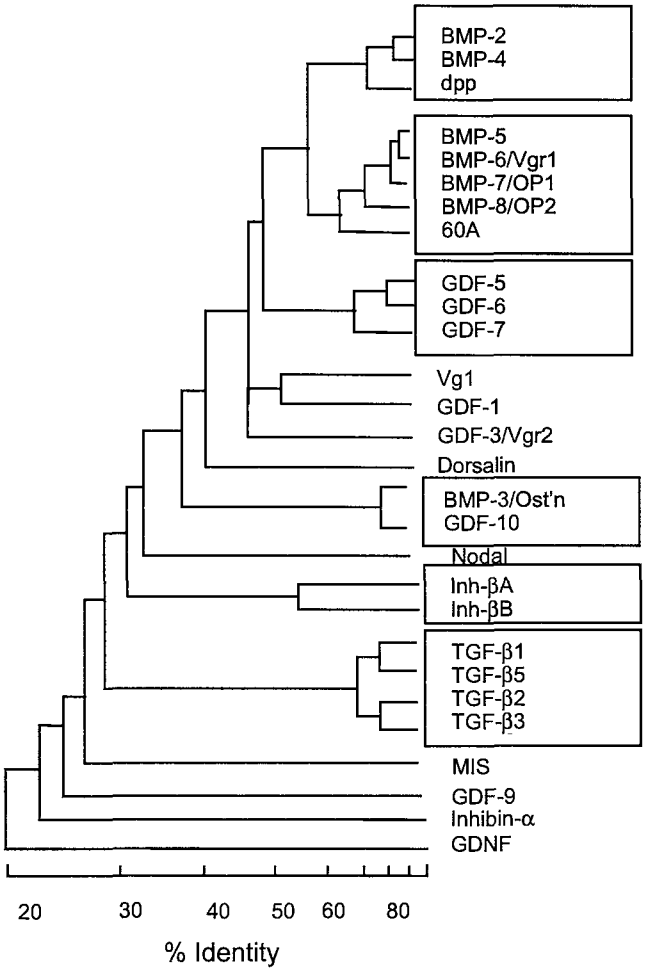


Fig 5.11. Dendogram of the TGFβ superfamily. All genes are mammalian except dpp and 60A from *Drosophila*, and Vg1 from *Xenopus* (Massague et al., 1994)
dpp- decapentaplegic, GDF- growth/differentiation factor
Vg- vitellogenin, MIS- Mullerian inhibiting substanance
GDNF- glia cell line-derived neurotrophic factor

5.10 Biological functions of activin A

Knockout mice lacking the inhibin βA chain had craniofacial abnormalities, lacked whiskers and their whisker follicles were abnormal. The mice died within 24 h of birth. This was because they failed to suckle due to a cleft palate (Matzuk et al., 1995a). The phenotype of these mice suggested that activin A was involved in formation of bone and possibly other tissues.

5.10.1 Activin plays a role in wound repair

Strong induction of activin A mRNA in the granulation tissue and hyper proliferative epithelium was found after skin injury in mice (Hubner et al., 1996). No activin A was detected in the skin of non-wounded mice. In mice overexpressing activin A in the skin (a transgene under a keratin 14 promoter), the subcutaneous fatty tissue was replaced by connective tissue and a severe thickening of the epidermis was found. After skin injury, a significant enhancement of granulation tissue formation was detected in the mice. *In situ* hybridisation revealed a strong expression of activin A mRNA in the hyperproliferative epithelium at the wound edge and in the epidermis adjacent to the wound (Munz et al., 1999). These findings suggested that activin may promote cell proliferation, especially during repair.

Attempts have been made to identify possible mediators of activin A induction during skin repair. Activin A and its mRNA are induced in cultured fibroblasts and keratinocytes upon stimulation with serum (Hubner and Werner, 1996). Platelet-poor plasma serum did not induce the protein. Therefore, most of the factors responsible for activin A induction by serum seemed to be derived from platelets, and were likely to be growth factors. TGFβ1, bFGF, PDGF and EGF all induced activin A mRNA in a dose-and time-dependent manner in fibroblasts. TGFβ1, EGF and keratinocyte growth factor induced activin A mRNA in keratinocytes. However, the signals were not as strong as those observed with serum, suggesting a combinatorial effect in the regulation of activin A.

Its expression is also stimulated by proinflammatory cytokines (Hubner and Werner, 1996). IL-1β, TNFα and IL-6 induced activin A in fibroblasts, while IL-1β and TNFα induced activin A in keratinocytes. TNFα gave the strongest response in both cell types.

5.10.2 Activin is a potent mesodermal inducer

Activin is a potent mesodermal inducer in *Xenopus*. Mesoderm induction begins at about the 64-cell stage, when signals from the vegetal hemisphere induce the equatorial region of the embryo to form mesoderm rather than ectoderm. This has been shown by juxtaposing tissue from the animal cap of the embryo, which is normally out of range of the mesoderm-inducing signal, with cells from the vegetal hemisphere. If the animal cap was cultured alone, it formed atypical epidermis. In the presence of vegetal pole cells, it formed a variety of mesodermal cell types such as muscle, notochord and blood. When the animal cap was cultured in the presence of activin, it also formed mesodermal cell types (Smith, 1993). Thomsen et al 90' showed that Xenopus animal caps treated with activin for two days developed into small embryoids with eyes and a cement gland at one end and a small tail-like structure on the other end. Melanocytes, notochord, neural tissue and muscle were also detected by histology that are specific mesodermal derived cell types. Untreated animal cap formed balls of epidermis. Activin mRNA was not detected in the egg, although transcripts encoding the protein are present from the mid-blastula stage. The mesodermal induction occurs before this, questioning the importance of activin as a mesodermal inducer in vivo. However significant amounts of activin-like activity have been extracted from the Xenopus egg and one alternative is that the activin is derived from the follicle cells surrounding the oocyte.

5.10.3 Activin A in cartilage and bone

Activin A enhances chondrogenesis in vitro in chicken limb bud micromass cultures (Jiang et al., 1993). The effect is concentration dependent. Activin A also induces a modest enhancement of type II collagen gene expression and proteoglycan synthesis by chondrocytes in micromass and explant cultures (Luyten et al., 1994). Activin and follistatin, are expressed during endochondral bone development. It has been suggested that activin is regulated by changes in the expression of follistatin at the stages of chondrogenesis and transition from cartilage to bone (Funaba et al.,

1996). Furthermore, activin was detected in proliferating chondrocytes and osteoblasts around fracture in a rat fibula fracture model. Local administration of activin promoted fracture healing in this model (Sakai et al., 1999).

5.11 The significance of activin in OA cartilage

Further investigation is needed to establish that activin production by OA cartilage is occurring in vivo and is not just a response to culture. It is also necessary to establish that it arises from the chondrocytes. When cartilage is dissected from the bone precautions are taken to avoid removing subchondral tissue or osteophytes, however it cannot be completely ruled out that any protein expressed could be derived from a "contaminating" tissue. This could be done by *in situ* hybridisation of activin mRNA in OA cartilage (i.e. without culture). Since activin A is a relatively small, secreted protein it would probably diffuse away into the joint cavity and synovial vasculature and so be difficult to detect directly in the tissue by immuno-histochemistry. If it can be established that activin is being made by chondrocytes in vivo, the next step would be to find out what stimulus is turning on its production in chondrocytes. Growth factors and proinflammatory cytokines would be obvious candidates. If a factor stimulating activin production by chondrocytes was present in the medium conditioned by OA explants, it could be purified and identified.

The effect of activin on chondrocyte metabolism needs to be studied. Overexpression of activin in the skin of transgenic mice lead to upregulation of fibronectin and tenascin-C expression compared to non-wounded mice. The levels of collagen were unchanged (Munz et al., 1999). Activin seems to differ in this respect from TGFβ, which is a strong inducer of collagen synthesis in many cell types including chondrocytes (Redini et al., 1988). It will be interesting to examine the effect of activin on expression of collagen, proteoglycans and fibronectin in chondrocytes.

Most of the non-OA samples produced little or no activin A, and its expression by cartilage from donors with osteomyelitis, avascular necrosis of bone or with an adjacent metastatic tumour may be a response to inflammation. Furthermore,

consistent with its relative absence from normal human cartilage, inhibin βA was not detected in porcine samples. It would be interesting to treat the human cartilage with follistatin or IRAP prior to 2D-analysis and study the effect on synthesis of activin A and possibly other proteins.

Cartilage from different joints was used in this study. It is not known to what extent cartilage from the femoral head and femoral condyle differ from cartilage of a non-weight bearing joint such as the humeral head in activin production. The humeral head cartilage may be more cellular, since smaller amounts generally secreted more protein than larger amounts of femoral cartilage. However this could also be due to increased cellular activity or to better quality cartilage. The 2D-gel patterns of cartilage explants from different joints were nevertheless qualitatively very similar.

5.12 Synthesis of type II collagen by OA cartilage

Type II collagen is the principal collagen in articular cartilage. Some labelled α-chain of type II collagen was detected on the 2D-gels, but due to its high molecular weight and basic pI it was not efficiently detected. In the explant medium samples the amount of type II collagen was estimated from the amount of labelled C-propeptide which focused well as a sharp spot. The C-propeptide of collagen type II was upregulated in OA samples compared with normal. In most of the normal samples the C-propeptide of collagen type II was not detected. The increased collagen synthesis in OA cartilage might be an attempt at repair.

Collagen synthesis, measured as [^{3}H]proline incorporation, has been reported to be increased in OA cartilage compared to normal (Lippiello et al., 1977). In 1993 Aigner et al showed expression of type II collagen mRNA in OA but not in normal cartilage using *in situ* hybridisation. This was subsequently confirmed by the same group using gene chip technology (Aigner et al., 2001). In contrast, Kumar et al, 2001 compared normal and OA cartilage by preparing cDNA libraries from mRNA. They found very low levels of collagen type II and no quantitative difference between normal and OA cartilage. The RNA used for their analyses came from pooled

cartilage from several patients (three for normal and five for OA). Thus individual differences between patients may not have been detected.

TGFβ is known to induce collagen gene expression. Generally the level of pro-inhibin βA and inhibin βA correlated with the level of the C-propeptide of collagen type II. Whether the increased collagen synthesis is induced by activin A or other mediators remains to be elucidated.

5.13 Role of YKL-40/gp-39 in human cartilage

Labelled YKL-40 was observed in 70 % (9/13) of the normal human explant media compared to 30 % (3/10) of the OA explant media samples. Kumar et al '2001 showed that YKL-40 is one of the most abundantly expressed genes in human articular cartilage, and was more abundant in OA than in normal cartilage. However differences between individual patients would not be detected due to pooling of cartilage. In another study, patients with late-stage OA had elevated levels of YKL-40 in serum and synovial fluid compared to healthy subjects (Johansen et al., 1996). The same group reported that spontaneous YKL-40 release was higher in OA than in normal cartilage explant cultures (Johansen et al., 2001). This is in disagreement with our preliminary data. It is possible that YKL-40 binds strongly to heparan sulphate or high molecular weight proteins that do not enter the 2D-gel and therefore avoids detection. In 1D-analysis of YKL-40 in normal and OA cartilage explant media however, the level of YKL-40 was similar (see *chapter 3*) suggesting this was not the case.

5.14 Expression of collagen type VI

Collagen type VI was expressed by human explant chondrocytes. The protein focused as an intense spot about 150 kDa. Due to its high abundance and the "burnt out" effect on the autoradiographs, it was not possible to determine if it was regulated. Pullig et al, 1999 reported that expression of type VI collagen mRNA was increased in the matrix of chondrocyte clusters and in the deep cartilage zone of severe OA

cartilage compared to normal. It was suggested that collagen type VI may function as a bridging molecule between the various components of the extracellular matrix and the chondrocyte surface (Pullig et al., 1999). Increased collagen type VI protein has been reported in OA cartilage compared to normal (Soder et al., 2002).

Chapter 6

Concluding discussion

6 Discussion

I have studied expression of proteins secreted by articular chondrocytes by separating them on 1D- or 2D-electrophoresis and identifying them with MS.

Porcine cartilage explants and chondrocytes were used to develop techniques to perform 2D-electrophoresis of secreted proteins, and to carry out initial experiments on regulation by IL-1.

Seven proteins that were regulated by IL-1 in monolayer chondrocytes were identified by 1D-electrophoresis and MS. These were MnSOD, SAA, TIMP-1 gp-38, MMP-1, MMP-3 and complement component C3. Comparison of proteins secreted by monolayer cells and cartilage explants showed that the constitutive protein expression varied with respect to the physiological surroundings of the cells, whereas proteins induced in response to IL-1 did not. However, differences were found in the response to IL-1 between porcine and human articular cells. In human cartilage, YKL40/gp-39 (the homologue of gp-38) was down-regulated by IL-1, and SAA was not detected.

As expected, some typical chondrocyte proteins were not detected and some proteins were found to co-migrate on the 1D-gel. To overcome the limited resolution of 1D, 2D-electrophoresis was performed. Preparing 2D-gels of proteins secreted by chondrocytes in explants was initially unsuccessful, because the proteins did not isoelectrically focus. This was resolved by removing proteoglycans from the explant culture medium by precipitation with CPC. A similar problem was encountered when performing 2D-electrophoresis of proteins secreted from monolayer cell cultures, although it did not seem to be due to the presence of aggrecan, but to some other polyanionic species. Removal of polyanionic molecules by passing the medium through an anion exchange column greatly improved the focusing, but this technique has not been optimised. It is possible that hyaluronan is responsible for this poor focusing but this remains to be confirmed.

Over 100 silver-stainable proteins were identified from porcine explant medium, but disappointingly no additional IL-1 regulated proteins were detected.

Once the technique to study proteins secreted from porcine cartilage explants was established, it was applied to human articular cartilage. So far, more than twenty metabolically labelled proteins secreted by cells in explants have been identified. This

number could be increased by loading more material on to the gels. To achieve this, samples from different patients could be pooled. More proteins could also be identified by analysing the CPC pellets since some may bind strongly to proteoglycans. Isoelectric focusing over a narrower pH gradient could be used to improve separation of proteins in crowded areas of the gel, as for instance around lumican and clusterin.

Many of the proteins detected in human cartilage were well known components of connective tissues. However, the production of relatively large amounts of activin by the OA cartilage was a novel finding. It was the only potentially regulatory molecule made in significant amounts and has not previously been detected in articular cartilage. The detection of increased type II collagen synthesis was also a novel finding and is in agreement with reports of increased mRNA for the protein in OA cartilage.

The potential regulatory molecules CTGF and cytokine-like protein C-17 were detected in some OA samples but their levels were relatively low and they could not be unambiguously identified in all gels. So it would be premature to conclude that their presence was associated with OA. However, CTGF is induced by TGFβ so it could be induced by activin.

Analysing more samples will hopefully provide information on whether YKL-40/gp-39 is down-regulated in OA or not.

There are difficulties quantifying proteins on 2D-gels. The silver stain has a limited dynamic range and while other more quantitative stains like Sypro Ruby are available, they require excising of spots under fluorescence light. Labelled proteins on the autoradiographs can be quantified but they are sometimes poorly resolved, and autoradiography also has a narrow dynamic range. In practise these problems are not of great importance, because the aim is to find proteins that show marked differences. Once identified, they can be quantified by methods such as enzyme-linked immunosorbent assay (ELISA). Initial work in our group has shown that activin levels measured by ELISA correlate well to the 2D-data.

6.1 Comparison of 2D-electrophoresis with other methods

An emerging technology that competes with or complements 2D-electrophoresis in proteomic analysis is multidimensional chromatography coupled to ESI MS/MS. Proteins are digested and the peptides loaded onto a chromatography column. The peptides can be separated using one or more on-line chromatography steps (Yates et al., 1999) prior to MS. In this technique there is no need for initial separation of the proteins. This is a useful technique to identify proteins in samples but it would be difficult to identify metabolically labelled proteins and to compare their amounts between different samples. Furthermore, it does not distinguish different forms and post translational modifications of proteins.

A more sophisticated chromatography based approach is to use isotope-coded affinity tags (ICAT) (Gygi et al., 1999b). Proteins in sample X are labelled on cysteine residues with a biotinylation reagent containing 8 deuterium atoms, whereas those in sample Y are labelled with a corresponding hydrogen containing reagent, resulting in an 8 Da mass difference between the derivatives. The two samples are mixed, proteins enzymatically digested and ICAT-labelled peptides are isolated on a biotin column. The labelled peptides are then separated and analysed by liquid chromatography coupled to MS/MS. Due to the 8 Da mass difference between the light and heavy isotope, the relative abundance of a peptide in sample X and Y can be compared.

Some problems have been encountered with this technique because the same peptide with a light or heavy isotope does not always co-elute and the presence of the derivative complicates the fragmentation pattern. It would be difficult to apply to the cartilage explants because newly synthesised material has to be distinguished from proteins leaching out from the tissue.

Differences in gene expression in cartilage could also be studied by using gene microarrays. This approach relies on obtaining 10-20 μg of good-quality mRNA per sample, so gram quantities of cartilage are required. Differences need to be verified by northern blotting. Changes in mRNA do not necessarily mean there will be

significant differences in the amounts of protein produced. Therefore separate studies of protein levels of candidate genes also need to be carried out.

Despite its drawbacks, the proteomic approach using 2D-electrophoresis is the simplest most direct way of studying secreted protein expression.

My work, as far as I am aware, is the first successful study of proteins secreted from cartilage explants using 2D-electrophoresis.

6.2 Comparison of protein expression in diseased and IL-1 treated cartilage

IL-1α and β have been reported in isolated human and bovine chondrocytes (Ollivierre et al., 1986) and increased IL-1β message has been described in OA cartilage (Attur et al., 1998). Both IL-1α and β protein have been detected in OA cartilage by immunohistochemistry (Towle et al., 1997) but the role the cytokine play in OA has been greatly debated. In this study most IL-1 stimulations were done on porcine cartilage and the OA tissue came from mature human cartilage.

Protein expression in porcine and human cartilage showed some similarities although the levels of expression of several proteins varied greatly. All human specimens except one were from adult donors whereas porcine material was obtained from 6 month old pigs (equivalent to human adolescent). Some differences in expression levels may therefore be attributable to this age difference (e.g. collagen type II). The changes in expression upon stimulation with IL-1 also differed between human and porcine cartilage. An overview of proteins detected in OA cartilage or induced by IL-1 stimulation is shown in table 6.1.

Table 6.1 Comparison of proteins synthesised by normal human, OA and porcine cartilage and the effect of IL-1 stimulation. Amounts of proteins are denoted by +/-. Proteins induced by IL-1 in human (normal and OA) and porcine cartilage are marked with an X.

Protein	Normal Human	OA	Normal Porcine	Induced by IL-1 in human	Induced by IL-1 in porcine
Collagen type II	-	++	+++++		
TIMP-1	++	++	+++		X
ASPIC	++	++	-		
Collagen type VI	++	++	-		
Clusterin	+++	+++	+		
Lumican	+++	+++	-		
YKL-39	++	++	-	X	
YKL-40/gp-39	+?	++?	-		X
MMP-1	-	++	-	X	X
MMP-3	-	++	-	X	X
Activin	-	+++	-		
MnSOD	-	-	-	X	X
Complement C3	-	-	-	X?	X
SAA	-	-	-		X

The C-terminal propeptide of collagen type II was the major stainable and metabolically labelled protein secreted into porcine explant medium. In contrast, healthy human cartilage produced very little collagen type II; however increased expression was obtained in OA cartilage.

The expression of the C-terminal propeptide and α-chain of collagen type II was sometimes down regulated upon IL-1 stimulation but this was not consistent. IL-1 has been reported to inhibit collagen type II synthesis by chondrocytes (Goldring et al., 1988).

Strong expression of TIMP-1 and osteonectin were always detected in both species. IL-1 increased the expression of TIMP-1 in porcine but not in human cartilage. Labelled ASPIC and collagen type VI were abundant in all human samples but were not detected in pigs. Some unlabelled collagen type VI was however detected in porcine cartilage. The glycoproteins clusterin and lumican were also strongly expressed in human cartilage but only clusterin was detected (unlabelled) in porcine. Lumican is absent in juvenile cartilage (Grover et al., 1995).

YKL-39 was a constant feature of the human explant medium patterns but was not detected in porcine samples. Labelled YKL-40/gp-39 was not always detected in human. YKL-40 expression was induced upon stimulation with IL-1 in porcine, but decreased in human cartilage. No correlation between YKL-40/gp-39 and OA has been established in this study. In contrast, other groups have reported an increase in YKL-40/gp-39 expression in OA (Johansen et al., 1996; Kumar et al., 2001). Johansen et al looked at the protein level in serum and synovial fluid and Kumar et al detected mRNA in a cartilage library.

MMP-1 and MMP-3 were detected in OA explant medium and their expression increased after stimulation with IL-1. In porcine cartilage, MMP-1 and MMP-3 were also induced upon stimulation with IL-1 but were not detected in untreated controls. The presence of MMPs in OA and the induction of MMPs by IL-1 are well documented in the literature (reviewed in Telow et al., 2001; Dinarello, 1991).

No activin was detected in porcine explant medium by 1D/2D-electrophoresis but it was highly expressed in human OA cartilage. IL-1 induces expression of activin

in human fibroblasts and keratinocytes (Hubner and Werner, 1996). However, I was unable to detect induction of activin in porcine chondrocytes by 1D-electrophoresis upon stimulation with IL-1. We are currently attempting to confirm this result by ELISA.

The mitochondrial protein MnSOD was induced by IL-1 in both human and porcine chondrocytes, but it was not was detected in control normal or OA cartilage extracts. MnSOD protects cells against reactive oxygen species that have been reported to play a role in RA (Mapp et al., 1995).

IL-1 also induced expression of complement component C3 and SAA in porcine. Protein bands that could correspond to complement C3, but not to SAA were detected in normal and OA human cartilage treated with IL-1. It is not known why SAA was not detected in human cartilage. Small levels of SAA have been suggested to be present in serum from patients with OA (Sipe, 1995). This is in contradiction to work by Sinz et al., 2002 (see section 6.2) that detected no SAA in serum or synovial fluid from patients with OA by 2D-electrophoresis and MS.

In conclusion, the proteins induced by IL-1 differ from the proteins secreted by OA cartilage except for the MMPs and YKL-39. Whether this means that IL-1 may not play a role in OA or that the OA cartilage is so late-stage that a primary IL-1 effect no longer is detectable remains to be elucidated.

6.3 Studies of gene expression in OA

Several groups have recently published studies on gene expression in OA cartilage. In Kumar et al., 2001, ESTs from adult human normal and OA cartilage cDNA libraries were identified. The libraries were prepared from total RNA and the ESTs sequenced and analysed using bioinformatic tools. Among the most abundant mRNAs identified from both libraries were decorin, COMP, plasma glutathione peroxidase, clusterin, CTGF and YKL-40/gp-39. The two latter proteins were more abundant in OA than normal cartilage. No YKL-39 was found. In contrast to my work MMP-3, TIMP-2, TIMP-3, TIMP-4 but not MMP-1 or TIMP-1 were detected at the

message level in both normal and OA cartilage. Collagen types I, II, VI, IX and XI were identified but not activin.

Aigner et al., 2001 performed a similar study using cDNA microarray technology. Normal, early-stage and late-stage OA cartilages were compared. Collagen type II and VI expression levels were increased in late stage OA compared to normal and early degenerated cartilage. Levels of fibronectin and osteonectin mRNA were increased in OA cartilage. No activin was detected but it is possible its cDNA was missing from the microarray used.

MMP-3 message was strongly expressed in normal and early-stage OA but was down regulated in late-stage OA. MMP-2 and MMP-11 were upregulated in late-stage OA suggesting that degradation pathways involving these MMPs may be more important in late-stage disease. TIMP-1, -3, -4-were detected in late-stage OA but as in the work by Kumar et al., 2001 no MMP-1 or TIMP-1 were found. It was surprising that MMP-1 and TIMP-1 were abundantly detected at the protein level by us but not at all found at the cDNA level by other groups. However, correlation between mRNA and protein levels is often poor (Gygi et al., 1999a).

More recently, the same group has performed studies of MMPs and aggrecanases in normal and OA cartilage using quantitative polymerase chain reaction assays (Bau et al., 2002). In accordance with the microarray result, the MMP-3 message was down-regulated in late-stage disease. A very small amount of MMP-1 was detected in all types of cartilage. MMP-13 (collagenase 3) expression was upregulated in late-stage OA. Levels of MMP-2 and MMP-11 were not measured.

The transcription of aggrecanase-1 (ADAMTS-4) was very low in all sample groups but increased slightly in late-stage OA. Aggrecanase-2 (ADAMTS-5) was constitutively expressed in all types of cartilage.

Sinz et al., 2002 compared protein expression in synovial fluid and plasma from patients with OA and RA using 2D-electrophoresis and MS. Fibrinogen β-chain degradation products were identified in both synovial fluid types but not in plasma. SAA protein spots were detected in synovial fluid and plasma from RA but not OA patients. Calgranulin B (a calcium-binding protein primarily expressed by circulating neutrophils and monocytes) was detected only in synovial fluid from patients with RA

and could perhaps be used as a diagnostic marker for arthritic diseases. (Sinz et al., 2002). We have detected unlabelled fibrinogen β-chain in OA explant medium samples but no SAA, which is in accordance with this study.

Many other groups have compared expression of specific proteins in normal and OA cartilage. For instance, Pullig et al., 1999 found an increased expression of collagen type VI protein and mRNA in OA cartilage compared to normal using immunochemistry. Increased expression of YKL-40/gp-39 protein was detected in serum and synovial fluid of patients with OA compared to normal subjects (Johansen et al., 1996). Messenger RNA and protein of Matrilin-3 are reported to be increased in OA articular cartilage compared to normal (Pullig et al., 2002). Matrilin-3 belongs to the matrilin family of extracellular matrix proteins containing von Willebrand factor A-like domains and is strongly expressed in growing bones. The protein was not detected by 2D-electrophoresis in this study.

Several academic research groups and pharmaceutical companies are currently using a proteomic approach to study chondrocyte expression, although very little has so far been published in peer-reviewed journals. Two recent publications using microarray technology are described below.

Stokes et al., 2002 used microarray analysis to compare gene expression in differentiated and dedifferentiated human fetal chondrocytes. The aim was to investigate the mechanism of cell dedifferentiation and how it relates to the phenotypic changes seen in chondrocytes during development of OA. The expression of 62 genes varied 2-fold or more between the two cell types. For instance, expression of collagen type IX and XI, aggrecan and COMP was down regulated and collagen type I, tenascin and cadherin 11 expression was upregulated in dedifferentiated cells after 11 days in culture.

Ibrahim et al., 2002 studied gene expression in inflamed paws from animals with collagen-induced arthritis. 223 genes showed differential expression between normal and diseased tissue, among them MMP-1, MMP-3 and TGFβ were increased. No activin or CTGF were detected in any of the studies.

6.4 Future work

In situ hybridisation of fresh tissue will be used to study activin expression by the chondrocytes in OA cartilage. This should reveal whether it is being made in diseased areas or adjacent normal tissue. The mechanisms underlying the increased synthesis of activin and collagen type II in OA need to be studied. Do chondrocytes isolated from OA cartilage maintain the expression or is it due to factors produced by the OA explants? If the latter, these factors could be purified. The effects of activin on cartilage metabolism also need to be investigated.

It would be valuable to be able to investigate chondrocyte protein secretion by small samples of cartilage. This would make it possible to study different areas of cartilage and small clinical samples. One way to achieve this would be to create an antibody array against all the proteins so far identified. Such an array could be used to quantify a large number of proteins in small samples. It could also be used to compare protein expression in cartilage from various areas of an articular surface, for analysing small pathological specimens of cartilage and for screening synovial fluid or sera for possible markers of OA.

Chapter 7

References

Abbaszade, I., Liu, R. Q., Yang, F., Rosenfeld, S. A., Ross, O. H., Link, J. R., Ellis, D. M., Tortorella, M. D., Pratta, M. A., Hollis, J. M., *et al.* (1999). Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family. J Biol Chem *274*, 23443-23450.

Aigner, T., Bertling, W., Stoss, H., Weseloh, G., and von der Mark, K. (1993). Independent expression of fibril-forming collagens I, II, and III in chondrocytes of human osteoarthritic cartilage. J Clin Invest *91*, 829-837.

Aigner, T., Hemmel, M., Neureiter, D., Gebhard, P. M., Zeiler, G., Kirchner, T., and McKenna, L. (2001). Apoptotic cell death is not a widespread phenomenon in normal aging and osteoarthritis human articular knee cartilage: a study of proliferation, programmed cell death (apoptosis), and viability of chondrocytes in normal and osteoarthritic human knee cartilage. Arthritis Rheum *44*, 1304-1312.

Aigner, T., Zien, A., Gehrsitz, A., Gebhard, P. M., and McKenna, L. (2001). Anabolic and catabolic gene expression pattern analysis in normal versus osteoarthritic cartilage using complementary DNA-array technology. Arthritis Rheum *44*, 2777-2789.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1994). Molecular Biology of the cell, third edn (New Yrok, Garland Publishing Inc.).

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res *25*, 3389-3402.

Attur, M. G., Patel, I. R., Patel, R. N., Abramson, S. B., and Amin, A. R. (1998). Autocrine production of IL-1 beta by human osteoarthritis-affected cartilage and differential regulation of endogenous nitric oxide, IL-6, prostaglandin E2, and IL-8. Proc Assoc Am Physicians *110*, 65-72.

Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G., and Yayon, A. (1994). Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis, and angiogenesis. Cell *79*, 1005-1013.

Aydelotte, M. B., Schumacher, B. L., and Kuettner, K. E. (1992). Heterogenity of articular chondrocytes. In Articular Cartilage and Osteoarthritis, K. E. Kuettner, R. Schleyerbach, J. G. Peyron, and V. C. Hascall, eds. (New York, Raven Press), pp. 237-249.

Azizan, A., Gaw, J. U., Govindraj, P., Tapp, H., and Neame, P. J. (2000). Chondromodulin I and pleiotrophin gene expression in bovine cartilage and epiphysis. Matrix Biol *19*, 521-531.

Badolato, R., and Oppenheim, J. J. (1996). Role of cytokines, acute-phase proteins, and chemokines in the progression of rheumatoid arthritis. [Review] [143 refs]. Semin Arthritis Rheum *26*, 526-538.

Barber, M., Bardoli, R. S., Segwick, R. D., and Tyler, A. N. (1981). Fast atom bombardment of solids as an ion source in mass spectrometry. Nature *293*, 270-275.

Bau, B., Gebhard, P. M., Haag, J., Knorr, T., Bartnik, E., and Aigner, T. (2002). Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. Arthritis Rheum *46*, 2648-2657.

Bayliss, M. T., Hutton, S., Hayward, J., and Maciewicz, R. A. (2001a). Distribution of aggrecanase (ADAMts 4/5) cleavage products in normal and osteoarthritic human articular cartilage: the influence of age, topography and zone of tissue. Osteoarthritis Cartilage *9*, 553-560.

Bayliss, M. T., Howat, S. L., Dudhia, J., Murphy, J. M., Barry, F. P., Edwards, J. C., and Day, A. J. (2001b). Up-regulation and differential expression of the hyaluronan-binding protein TSG-6 in cartilage and synovium in rheumatoid arthritis and osteoarthritis. Osteoarthritis Cartilage *9*, 42-48.

Beavis, R. C., Chaudhary, T., and Chait, B. T. (1992). □-Cyano-4-hydroxycinnamic acid as a matrix for matrix-assisted laser desorption mass spectrometry. Organic mass spectrometry *27*, 156-158.

Benya, P. D., Padilla, S. R., and Nimni, M. E. (1977). The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II. Verifications by cyanogen bromide peptide analysis. Biochemistry *16*, 865-872.

Bernard, D. J., Chapman, S. C., and Woodruff, T. K. (2001). Mechanisms of inhibin signal transduction. Recent Prog Horm Res *56*, 417-450.

Billington, C. J., Clark, I. M., and Cawston, T. E. (1998). An aggrecan-degrading activity associated with chondrocyte membranes. Biochem J *336 (Pt 1)*, 207-212.

Bjellqvist, B., Ek, K., Righetti, P. G., Gianazza, E., Gorg, A., Westermeier, R., and Postel, W. (1982). Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J Biochem Biophys Methods *6*, 317-339.

Blanco, F. J., Ochs, R. L., Schwarz, H., and Lotz, M. (1995). Chondrocyte apoptosis induced by nitric oxide. Am J Pathol *146*, 75-85.

Borman, S. (1998). A brief history of mass spectrometry (http://masspec.scripps.edu/Hist-ms.htm).

Boumediene, K., Conrozier, T., Mathieu, P., Richard, M., Marcelli, C., Vignon, E., and Pujol, J. P. (1998). Decrease of cartilage transforming growth factor-beta receptor II expression in the rabbit experimental osteoarthritis--potential role in cartilage breakdown. Osteoarthritis Cartilage *6*, 146-149.

Bos, P. K., van Osch, G. J., Frenz, D. A., Verhaar, J. A., and Verwoerd-Verhoef, H. L. (2001). Growth factor expression in cartilage wound healing: temporal and spatial immunolocalization in a rabbit auricular cartilage wound model. Osteoarthritis Cartilage *9*, 382-389.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*, 248-254.

Brew, K., Dinakarpandian, D., and Nagase, H. (2000). Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta *1477*, 267-283.

Buckwalter, J. A., and Hunziker, E. B. (2000). Articular cartilage morphology and biology. In Biology of the Synovial Joint, C. Archer, B. Caterson, M. Benjamin, and J. Ralphs, eds., pp. 75-100.

Byers, P. D., Maroudase, A., Oztop, F., Stockwell, R. A., and Venn, M. F. (1977). Histological and biochemical studies on cartilage from osteoarthrotic femoral heads with special reference to surface characteristics. Connect Tissue Res *5*, 41-49.

Chambers, M. G., Bayliss, M. T., and Mason, R. M. (1997). Chondrocyte cytokine and growth factor expression in murine osteoarthritis. Osteoarthritis Cartilage *5*, 301-308.

Chandrasekhar, S., and Harvey, A. K. (1988). Transforming growth factor-beta is a potent inhibitor of IL-1 induced protease activity and cartilage proteoglycan degradation. Biochem Biophys Res Commun *157*, 1352-1359.

Chandrasekhar, S., Harvey, A. K., Johnson, M. G., and Becker, G. W. (1994). Osteonectin/SPARC is a product of articular chondrocytes/cartilage and is regulated by cytokines and growth factors. Biochim Biophys Acta *1221*, 7-14.

Chang, S. C., Hoang, B., Thomas, J. T., Vukicevic, S., Luyten, F. P., Ryba, N. J., Kozak, C. A., Reddi, A. H., and Moos, M., Jr. (1994). Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. J Biol Chem *269*, 28227-28234.

Chen, P., Yu, Y. M., and Reddi, A. H. (1993). Chondrogenesis in chick limb bud mesodermal cells: reciprocal modulation by activin and inhibin. Exp Cell Res *206*, 119-127.

Chong, H., Pangas, S. A., Bernard, D. J., Wang, E., Gitch, J., Chen, W., Draper, L. B., Cox, E. T., and Woodruff, T. K. (2000). Structure and expression of a membrane component of the inhibin receptor system. Endocrinology *141*, 2600-2607.

Clauser, K. R., Hall, S. C., Smith, D. M., Webb, J. W., Andrews, L. E., Tran, H. M., Epstein, L. B., and Burlingame, A. L. (1995). Rapid mass spectrometric peptide sequencing and mass matching for characterization of human melanoma proteins isolated by two-dimensional PAGE. Proc Natl Acad Sci U S A *92*, 5072-5076.

Collins, D. (1949). The Pathology of Articular and Spinal diseases (London, Edward Arnold & CO.).

Comper, W. (1991). Physiochemical aspects of cartilage extracellular matrix. In Cartilage: Molecular Aspects, B. Hall, and S. Newman, eds. (Boca Raton, FL, CRC Press), pp. 59-96.

Connor, J. R., Kumar, S., Sathe, G., Mooney, J., O'Brien, S. P., Mui, P., Murdock, P. R., Gowen, M., and Lark, M. W. (2001). Clusterin expression in adult human normal and osteoarthritic articular cartilage. Osteoarthritis Cartilage *9*, 727-737.

Costell, M., Gustafsson, E., Aszodi, A., Morgelin, M., Bloch, W., Hunziker, E., Addicks, K., Timpl, R., and Fassler, R. (1999). Perlecan maintains the integrity of cartilage and some basement membranes. J Cell Biol *147*, 1109-1122.

Crawford, J., and Campbell, M. D. (1969). The healing of cartilage defects. Clinical orthopaedics and related research *64*, 45-63.

Cunnane, G. (2001). Amyloid precursors and amyloidosis in inflammatory arthritis. Curr Opin Rheumatol *13*, 67-73.

Cunnane, G., Grehan, S., Geoghegan, S., McCormack, C., Shields, D., Whitehead, A. S., Bresnihan, B., and Fitzgerald, O. (2000). Serum amyloid A in the assessment of early inflammatory arthritis. J Rheumatol *27*, 58-63.

Del Carlo, M., Jr., and Loeser, R. F. (2002). Nitric oxide-mediated chondrocyte cell death requires the generation of additional reactive oxygen species. Arthritis Rheum *46*, 394-403.

Del Rosso, M., Fibbi, G., and Matucci Cerinic, M. (1999). The urokinase-type plasminogen activator system and inflammatory joint diseases. Clin Exp Rheumatol *17*, 485-498.

Dinarello, C. A. (1991). Interleukin-1 and interleukin-1 antagonism. Blood *77*, 1627-1652.

Dore, S., Pelletier, J. P., DiBattista, J. A., Tardif, G., Brazeau, P., and Martel-Pelletier, J. (1994). Human osteoarthritic chondrocytes possess an increased number of insulin-like growth factor 1 binding sites but are unresponsive to its stimulation. Possible role of IGF-1-binding proteins. Arthritis Rheum *37*, 253-263.

Ducret, A., Bruun, C. F., Bures, E. J., Marhaug, G., Husby, G., and Aebersold, R. (1996). Characterization of human serum amyloid A protein isoforms separated by two-dimensional electrophoresis by liquid chromatography/electrospray ionization tandem mass spectrometry. Electrophoresis *17*, 866-876.

Dudley, A. T., Lyons, K. M., and Robertson, E. J. (1995). A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. Genes Dev *9*, 2795-2807.

Erlacher, L., Ng, C. K., Ullrich, R., Krieger, S., and Luyten, F. P. (1998). Presence of cartilage-derived morphogenetic proteins in articular cartilage and enhancement of matrix replacement in vitro. Arthritis Rheum *41*, 263-273.

Farndale, R. W., Sayers, C. A., and Barrett, A. J. (1982). A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. Connect Tissue Res *9*, 247-248.

Feldmann, M., Brennan, F. M., and Maini, R. N. (1996). Rheumatoid arthritis. Cell *85*, 307-310.

Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., and Whitehouse, C. M. (1989). Electrospray ionization for mass spectrometry of large biomolecules. Science *246*, 64-71.

Ferrell, W. R., and Najafipour, H. (1992). Changes in synovial PO2 and blood flow in the rabbit knee joint due to stimulation of the posterior articular nerve. J Physiol *449*, 607-617.

Flannery, C. R., Lark, M. W., and Sandy, J. D. (1992). Identification of a stromelysin cleavage site within the interglobular domain of human aggrecan. Evidence for proteolysis at this site in vivo in human articular cartilage. J Biol Chem *267*, 1008-1014.

Flechtenmacher, J., Huch, K., Thonar, E. J., Mollenhauer, J. A., Davies, S. R., Schmid, T. M., Puhl, W., Sampath, T. K., Aydelotte, M. B., and Kuettner, K. E. (1996). Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum *39*, 1896-1904.

Fortier, L. A., Balkman, C. E., Sandell, L. J., Ratcliffe, A., and Nixon, A. J. (2001). Insulin-like growth factor-I gene expression patterns during spontaneous repair of acute articular cartilage injury. J Orthop Res *19*, 720-728.

Fosang, A. J., Last, K., Fujii, Y., Seiki, M., and Okada, Y. (1998). Membrane-type 1 MMP (MMP-14) cleaves at three sites in the aggrecan interglobular domain. FEBS Lett *430*, 186-190.

Fosang, A. J., Last, K., Knauper, V., Murphy, G., and Neame, P. J. (1996). Degradation of cartilage aggrecan by collagenase-3 (MMP-13). FEBS Lett *380*, 17-20.

Fosang, A. J., Last, K., Knauper, V., Neame, P. J., Murphy, G., Hardingham, T. E., Tschesche, H., and Hamilton, J. A. (1993). Fibroblast and neutrophil collagenases cleave at two sites in the cartilage aggrecan interglobular domain. Biochem J *295 (Pt 1)*, 273-276.

Fosang, A. J., Neame, P. J., Last, K., Hardingham, T. E., Murphy, G., and Hamilton, J. A. (1992). The interglobular domain of cartilage aggrecan is cleaved by PUMP, gelatinases, and cathepsin B. J Biol Chem *267*, 19470-19474.

Frenkel, S. R., Clancy, R. M., Ricci, J. L., Di Cesare, P. E., Rediske, J. J., and Abramson, S. B. (1996). Effects of nitric oxide on chondrocyte migration, adhesion, and cytoskeletal assembly. Arthritis Rheum *39*, 1905-1912.

Froesch, E. R., Schmid, C., Schwander, J., and Zapf, J. (1985). Actions of insulin-like growth factors. Annu Rev Physiol *47*, 443-467.

Fujimoto, E., Ochi, M., Kato, Y., Mochizuki, Y., Sumen, Y., and Ikuta, Y. (1999). Beneficial effect of basic fibroblast growth factor on the repair of full-thickness defects in rabbit articular cartilage. Arch Orthop Trauma Surg *119*, 139-145.

Funaba, M., Ogawa, K., Murata, T., Fujimura, H., Murata, E., Abe, M., Takahashi, M., and Torii, K. (1996). Follistatin and activin in bone: expression and localization during endochondral bone development. Endocrinology *137*, 4250-4259.

Garcia-Ramirez, M., Toran, N., Andaluz, P., Carrascosa, A., and Audi, L. (2000). Vascular endothelial growth factor is expressed in human fetal growth cartilage. J Bone Miner Res *15*, 534-540.

Gerber, H. P., Vu, T. H., Ryan, A. M., Kowalski, J., Werb, Z., and Ferrara, N. (1999). VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med *5*, 623-628.

Gharahdaghi, F., Weinberg, C. R., Meagher, D. A., Imai, B. S., and Mische, S. M. (1999). Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. Electrophoresis *20*, 601-605.

Glansbeek, H. L., van Beuningen, H. M., Vitters, E. L., Morris, E. A., van der Kraan, P. M., and van den Berg, W. B. (1997). Bone morphogenetic protein 2 stimulates articular cartilage proteoglycan synthesis in vivo but does not counteract interleukin-1alpha effects on proteoglycan synthesis and content. Arthritis Rheum *40*, 1020-1028.

Goldring, M. B., Birkhead, J., Sandell, L. J., Kimura, T., and Krane, S. M. (1988). Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. J Clin Invest *82*, 2026-2037.

Grover, J., Chen, X. N., Korenberg, J. R., and Roughley, P. J. (1995). The human lumican gene. Organization, chromosomal location, and expression in articular cartilage. J Biol Chem *270*, 21942-21949.

Gygi, S. P., Rochon, Y., Franza, B. R., and Aebersold, R. (1999a). Correlation between protein and mRNA abundance in yeast. Mol Cell Biol *19*, 1720-1730.

Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999b). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol *17*, 994-999.

Hagmann, M. (1999). New leads to cancer, arthritis therapies. Science *284*, 1600-1601.

Hakala, B. E., White, C., and Recklies, A. D. (1993). Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. J Biol Chem *268*, 25803-25810.

Hardingham, T. E., and Fosang, A. J. (1992). Proteoglycans: many forms and many functions. Faseb J *6*, 861-870.

Hardingham, T. E., Bayliss, M. T., Rayan, V., and Noble, D. P. (1992). Effects of growth factors and cytokines on proteoglycan turnover in articular cartilage. Br J Rheumatol *31 Suppl 1*, 1-6.

Harvey, A. K., Stack, S. T., and Chandrasekhar, S. (1993). Differential modulation of degradative and repair responses of interleukin-1-treated chondrocytes by platelet-derived growth factor. Biochem J *292 (Pt 1)*, 129-136.

Hashimoto, S., Setareh, M., Ochs, R. L., and Lotz, M. (1997). Fas/Fas ligand expression and induction of apoptosis in chondrocytes. Arthritis Rheum *40*, 1749-1755.

Hashimoto, S., Ochs, R. L., Komiya, S., and Lotz, M. (1998a). Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. Arthritis Rheum *41*, 1632-1638.

Hashimoto, S., Takahash, K., Amiel, D., Coutts, R. D., and Lotz, M. (1998b). Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. Arthritis Rheum *41*, 1266-1274.

Hedbom, E., Antonsson, P., Hjerpe, A., Aeschlimann, D., Paulsson, M., Rosa-Pimentel, E., Sommarin, Y., Wendel, M., Oldberg, A., and Heinegard, D. (1992). Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. J Biol Chem *267*, 6132-6136.

Hedger, M. P., Drummond, A. E., Robertson, D. M., Risbridger, G. P., and de Kretser, D. M. (1989). Inhibin and activin regulate [3H]thymidine uptake by rat thymocytes and 3T3 cells in vitro. Mol Cell Endocrinol *61*, 133-138.

Heinegard, D., and Oldberg, A. (1989). Structure and biology of cartilage and bone matrix noncollagenous macromolecules. Faseb J *3*, 2042-2051.

Henderson, B., Thompson, R. C., Hardingham, T., and Lewthwaite, J. (1991). Inhibition of interleukin-1-induced synovitis and articular cartilage proteoglycan loss in the rabbit knee by recombinant human interleukin-1 receptor antagonist. Cytokine *3*, 246-249.

Hildebrand, A., Romaris, M., Rasmussen, L. M., Heinegard, D., Twardzik, D. R., Border, W. A., and Ruoslahti, E. (1994). Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochem J *302 (Pt 2)*, 527-534.

Hiraki, Y., Inoue, H., Iyama, K., Kamizono, A., Ochiai, M., Shukunami, C., Iijima, S., Suzuki, F., and Kondo, J. (1997). Identification of chondromodulin I as a novel endothelial cell growth inhibitor. Purification and its localization in the avascular zone of epiphyseal cartilage. J Biol Chem *272*, 32419-32426.

Hiraki, Y., Inoue, H., Kondo, J., Kamizono, A., Yoshitake, Y., Shukunami, C., and Suzuki, F. (1996). A novel growth-promoting factor derived from fetal bovine cartilage, chondromodulin II. Purification and amino acid sequence. J Biol Chem *271*, 22657-22662.

Hiraki, Y., Tanaka, H., Inoue, H., Kondo, J., Kamizono, A., and Suzuki, F. (1991). Molecular cloning of a new class of cartilage-specific matrix, chondromodulin-I, which stimulates growth of cultured chondrocytes. Biochem Biophys Res Commun *175*, 971-977.

Hogan, B. L. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev *10*, 1580-1594.

Hojima, Y., van der Rest, M., and Prockop, D. J. (1985). Type I procollagen carboxyl-terminal proteinase from chick embryo tendons. Purification and characterization. J Biol Chem *260*, 15996-16003.

Hollander, A. P., Pidoux, I., Reiner, A., Rorabeck, C., Bourne, R., and Poole, A. R. (1995). Damage to type II collagen in aging and osteoarthritis starts at the articular surface, originates around chondrocytes, and extends into the cartilage with progressive degeneration. J Clin Invest *96*, 2859-2869.

Homandberg, G. A. (1999). Potential regulation of cartilage metabolism in osteoarthritis by fibronectin fragments. Front Biosci *4*, D713-730.

Homandberg, G. A., and Hui, F. (1994). High concentrations of fibronectin fragments cause short-term catabolic effects in cartilage tissue while lower concentrations cause continuous anabolic effects. Arch Biochem Biophys *311*, 213-218.

Homandberg, G. A., Hui, F., Wen, C., Purple, C., Bewsey, K., Koepp, H., Huch, K., and Harris, A. (1997). Fibronectin-fragment-induced cartilage chondrolysis is associated with release of catabolic cytokines. Biochem J *321*, 751-757.

Homandberg, G. A., Meyers, R., and Xie, D. L. (1992). Fibronectin fragments cause chondrolysis of bovine articular cartilage slices in culture. J Biol Chem *267*, 3597-3604.

Hu, B., Trinh, K., Figueira, W. F., and Price, P. A. (1996). Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. J Biol Chem *271*, 19415-19420.

Hubner, G., and Werner, S. (1996). Serum growth factors and proinflammatory cytokines are potent inducers of activin expression in cultured fibroblasts and keratinocytes. Exp Cell Res *228*, 106-113.

Hubner, G., Hu, Q., Smola, H., and Werner, S. (1996). Strong induction of activin expression after injury suggests an important role of activin in wound repair. Dev Biol *173*, 490-498.

Huch, K., Wilbrink, B., Flechtenmacher, J., Koepp, H. E., Aydelotte, M. B., Sampath, T. K., Kuettner, K. E., Mollenhauer, J., and Thonar, E. J. (1997). Effects of recombinant human osteogenic protein 1 on the production of proteoglycan, prostaglandin E2, and interleukin-1 receptor antagonist by human articular chondrocytes cultured in the presence of interleukin-1beta. Arthritis Rheum *40*, 2157-2161.

Ibrahim, S. M., Koczan, D., and Thiesen, H. J. (2002). Gene-expression profile of collagen-induced arthritis. J Autoimmun *18*, 159-167.

Iozzo, R. V., Cohen, I. R., Grassel, S., and Murdoch, A. D. (1994). The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices. Biochem J *302 (Pt 3)*, 625-639.

Jeno, P., Mini, T., Moes, S., Hintermann, E., and Horst, M. (1995). Internal sequences from proteins digested in polyacrylamide gels. Anal Biochem *224*, 75-82.

Jensen, O. N., Podtelejnikov, A., and Mann, M. (1996). Delayed extraction improves specificity in database searches by matrix- assisted laser desorption/ionization peptide maps. Rapid Commun Mass Spectrom *10*, 1371-1378.

Jiang, T. X., Yi, J. R., Ying, S. Y., and Chuong, C. M. (1993). Activin enhances chondrogenesis of limb bud cells: stimulation of precartilaginous mesenchymal condensations and expression of NCAM. Dev Biol *155*, 545-557.

Jin, D. Y., Lyu, M. S., Kozak, C. A., and Jeang, K. T. (1996). Function of 14-3-3 proteins. Nature *382*, 308.

Johansen, J. S., Hvolris, J., Hansen, M., Backer, V., Lorenzen, I., and Price, P. A. (1996). Serum YKL-40 levels in healthy children and adults. Comparison with serum and synovial fluid levels of YKL-40 in patients with osteoarthritis or trauma of the knee joint. Br J Rheumatol *35*, 553-559.

Johansen, J. S., Olee, T., Price, P. A., Hashimoto, S., Ochs, R. L., and Lotz, M. (2001). Regulation of YKL-40 production by human articular chondrocytes. Arthritis Rheum *44*, 826-837.

Kaps, C., Bramlage, C., Smolian, H., Haisch, A., Ungethum, U., Burmester, G. R., Sittinger, M., Gross, G., and Haupl, T. (2002). Bone morphogenetic proteins promote cartilage differentiation and protect engineered artificial cartilage from fibroblast invasion and destruction. Arthritis Rheum *46*, 149-162.

Karas, M., and Hillenkamp, F. (1988). Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem *60*, 2299-2301.

Kashiwagi, M., Tortorella, M., Nagase, H., and Brew, K. (2001). TIMP-3 is a potent inhibitor of aggrecanase 1 (ADAM-TS4) and aggrecanase 2 (ADAM-TS5). J Biol Chem *276*, 12501-12504.

Keele, B. B., Jr., McCord, J. M., and Fridovich, I. (1970). Superoxide dismutase from escherichia coli B. A new manganese- containing enzyme. J Biol Chem *245*, 6176-6181.

Kirkpatrick, R. B., Emery, J. G., Connor, J. R., Dodds, R., Lysko, P. G., and Rosenberg, M. (1997). Induction and expression of human cartilage glycoprotein 39 in rheumatoid inflammatory and peripheral blood monocyte-derived macrophages. Exp Cell Res *237*, 46-54.

Klagsbrun, M., and D'Amore, P. A. (1996). Vascular endothelial growth factor and its receptors. Cytokine Growth Factor Rev *7*, 259-270.

Klagsburn, M., Langer, R., Levenson, R., Smith, S., and Lillehei, C. (1977). The stimulation of DNA synthesis and cell division in chondrocytes and 3T3 cells by a growth factor isolated from cartilage. Exp Cell Res *105*, 99-108.

Klose, J. (1975). Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals. Humangenetik *26*, 231-243.

Kluve-Beckerman, B., Drumm, M. L., and Benson, M. D. (1991). Nonexpression of the human serum amyloid A three (SAA3) gene. DNA Cell Biol *10*, 651-661.

Kouri, J. B., Aguilera, J. M., Reyes, J., Lozoya, K. A., and Gonzalez, S. (2000). Apoptotic chondrocytes from osteoarthrotic human articular cartilage and abnormal calcification of subchondral bone. J Rheumatol *27*, 1005-1019.

Kumar, S., Connor, J. R., Dodds, R. A., Halsey, W., Van Horn, M., Mao, J., Sathe, G., Mui, P., Agarwal, P., Badger, A. M., *et al.* (2001). Identification and initial characterization of 5000 expressed sequenced tags (ESTs) each from adult human normal and osteoarthritic cartilage cDNA libraries. Osteoarthritis Cartilage *9*, 641-653.

Lacraz, S., Nicod, L. P., Chicheportiche, R., Welgus, H. G., and Dayer, J. M. (1995). IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human mononuclear phagocytes. J Clin Invest *96*, 2304-2310.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lammi, M. J., Inkinen, R., Parkkinen, J. J., Hakkinen, T., Jortikka, M., Nelimarkka, L. O., Jarvelainen, H. T., and Tammi, M. I. (1994). Expression of reduced amounts of structurally altered aggrecan in articular cartilage chondrocytes exposed to high hydrostatic pressure. Biochem J *304 (Pt 3)*, 723-730.

Lane, T. F., and Sage, E. H. (1994). The biology of SPARC, a protein that modulates cell-matrix interactions. Faseb J *8*, 163-173.

Lark, M. W., Bayne, E. K., Flanagan, J., Harper, C. F., Hoerrner, L. A., Hutchinson, N. I., Singer, II, Donatelli, S. A., Weidner, J. R., Williams, H. R., *et al.* (1997). Aggrecan degradation in human cartilage. Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. J Clin Invest *100*, 93-106.

Laurent, T., and Scott, J. E. (1964). Molecular weight fractionation of polyanions by cetylpyridinium chloride in salt solutions. Nature *202*, 661-662.

Lee, R. B., Wilkins, R. J., Razaq, S., and Urban, J. P. (2002). The effect of mechanical stress on cartilage energy metabolism. Biorheology *39*, 133-143.

Lee, T. H., Wisniewski, H. G., and Vilcek, J. (1992). A novel secretory tumor necrosis factor-inducible protein (TSG-6) is a member of the family of hyaluronate binding proteins, closely related to the adhesion receptor CD44. J Cell Biol *116*, 545-557.

Lewis, K. A., Gray, P. C., Blount, A. L., MacConell, L. A., Wiater, E., Bilezikjian, L. M., and Vale, W. (2000). Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. Nature *404*, 411-414.

Lietman, S. A., Yanagishita, M., Sampath, T. K., and Reddi, A. H. (1997). Stimulation of proteoglycan synthesis in explants of porcine articular cartilage by recombinant osteogenic protein-1 (bone morphogenetic protein-7). J Bone Joint Surg Am *79*, 1132-1137.

Lippiello, L., Hall, D., and Mankin, H. J. (1977). Collagen synthesis in normal and osteoarthritic human cartilage. J Clin Invest *59*, 593-600.

Liu, X., Rapp, N., Deans, R., and Cheng, L. (2000). Molecular cloning and chromosomal mapping of a candidate cytokine gene selectively expressed in human CD34+ cells. Genomics *65*, 283-292.

Lobb, R., Sasse, J., Sullivan, R., Shing, Y., D'Amore, P., Jacobs, J., and Klagsbrun, M. (1986). Purification and characterization of heparin-binding endothelial cell growth factors. J Biol Chem *261*, 1924-1928.

Lohmander, L. S., Ionescu, M., Jugessur, H., and Poole, A. R. (1999). Changes in joint cartilage aggrecan after knee injury and in osteoarthritis. Arthritis Rheum *42*, 534-544.

Lohmander, L. S., Neame, P. J., and Sandy, J. D. (1993). The structure of aggrecan fragments in human synovial fluid. Evidence that aggrecanase mediates cartilage degradation in inflammatory joint disease, joint injury, and osteoarthritis. Arthritis Rheum *36*, 1214-1222.

Lorenzo, P., Bayliss, M. T., and Heinegard, D. (1998a). A novel cartilage protein (CILP) present in the mid-zone of human articular cartilage increases with age. J Biol Chem *273*, 23463-23468.

Lorenzo, P., Neame, P., Sommarin, Y., and Heinegard, D. (1998b). Cloning and deduced amino acid sequence of a novel cartilage protein (CILP) identifies a proform including a nucleotide pyrophosphohydrolase. J Biol Chem *273*, 23469-23475.

Lum, Z. P., Hakala, B. E., Mort, J. S., and Recklies, A. D. (1996). Modulation of the catabolic effects of interleukin-1 beta on human articular chondrocytes by transforming growth factor-beta. J Cell Physiol *166*, 351-359.

Luyten, F. P., Chen, P., Paralkar, V., and Reddi, A. H. (1994). Recombinant bone morphogenetic protein-4, transforming growth factor-beta 1, and activin A enhance the cartilage phenotype of articular chondrocytes in vitro. Exp Cell Res *210*, 224-229.

Luyten, F. P., Yu, Y. M., Yanagishita, M., Vukicevic, S., Hammonds, R. G., and Reddi, A. H. (1992). Natural bovine osteogenin and recombinant human bone morphogenetic protein-2B are equipotent in the maintenance of proteoglycans in bovine articular cartilage explant cultures. J Biol Chem *267*, 3691-3695.

Macfarlane, R. D., and Torgerson, D. F. (1976). Californium-252 plasma desorption mass spectroscopy. Science *191*, 920-925.

Mackey, A. J., Haystead, T. A., and Pearson, W. R. (2002). Getting more from less: algorithms for rapid protein identification with multiple short Peptide sequences. Mol Cell Proteomics *1*, 139-147.

Mali, M., Jaakkola, P., Arvilommi, A. M., and Jalkanen, M. (1990). Sequence of human syndecan indicates a novel gene family of integral membrane proteoglycans. J Biol Chem *265*, 6884-6889.

Mamyrin, B. A. (1994). Laser assited reflectron time-of-flight mass spectrometry. Int J Mass Spectrom Ion Processes *131*, 1-19.

Mankin, H. J., and Lippiello, L. (1971). The glycosaminoglycans of normal and arthritic cartilage. J Clin Invest *50*, 1712-1719.

Mankin, H., and Brandt, K. (1997). Pathogenesis of osteoarthritis. In Textbook of Rheumatology, W. Kelley, E. Harris, S. Ruddy, and C. Sledge, eds. (Philadelphia, W.B. Saunders Company), pp. 1369-1381.

Mann, M. (1996). A shortcut to interesting human genes: peptide sequence tags, expressed- sequence tags and computers. Trends Biochem Sci *21*, 494-495.

Mann, M., and Talbo, G. (1996). Developments in matrix-assisted laser desorption/ionization peptide mass spectrometry. Curr Opin Biotechnol *7*, 11-19.

Mann, M., and Wilm, M. (1995). Electrospray mass spectrometry for protein characterization. Trends Biochem Sci *20*, 219-224.

Mann, M., Hojrup, P., and Roepstorff, P. (1993). Use of mass spectrometric molecular weight information to identify proteins in sequence databases. Biol Mass Spectrom *22*, 338-345.

Mapp, P. I., Grootveld, M. C., and Blake, D. R. (1995). Hypoxia, oxidative stress and rheumatoid arthritis. Br Med Bull *51*, 419-436.

Massague, J., Attisano, L., and Wrana, J. L. (1994). The TGF-□ family and its composite receptors. Trends in Cell Biology *4*, 172-178.

Mathews, L. S., and Vale, W. W. (1991). Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell *65*, 973-982.

Matzuk, M. M., Kumar, T. R., Vassalli, A., Bickenbach, J. R., Roop, D. R., Jaenisch, R., and Bradley, A. (1995a). Functional analysis of activins during mammalian development. Nature *374*, 354-356.

Matzuk, M. M., Lu, N., Vogel, H., Sellheyer, K., Roop, D. R., and Bradley, A. (1995b). Multiple defects and perinatal death in mice deficient in follistatin. Nature *374*, 360-363.

Mengshol, J. A., Mix, K. S., and Brinckerhoff, C. E. (2002). Matrix metalloproteinases as therapeutic targets in arthritic diseases: bull's-eye or missing the mark? Arthritis Rheum *46*, 13-20.

Meredith, J. E., Jr., Fazeli, B., and Schwartz, M. A. (1993). The extracellular matrix as a cell survival factor. Mol Biol Cell *4*, 953-961.

Merino, R., Macias, D., Ganan, Y., Rodriguez-Leon, J., Economides, A. N., Rodriguez-Esteban, C., Izpisua-Belmonte, J. C., and Hurle, J. M. (1999). Control of digit formation by activin signalling. Development *126*, 2161-2170.

Moon, R., Parikh, A. A., Szabo, C., Fischer, J. E., Salzman, A. L., and Hasselgren, P. O. (1997). Complement C3 production in human intestinal epithelial cells is regulated by interleukin 1beta and tumor necrosis factor alpha. Arch Surg *132*, 1289-1293.

Moussad, E. E., and Brigstock, D. R. (2000). Connective tissue growth factor: what's in a name? Mol Genet Metab *71*, 276-292.

Munz, B., Smola, H., Engelhardt, F., Bleuel, K., Brauchle, M., Lein, I., Evans, L. W., Huylebroeck, D., Balling, R., and Werner, S. (1999). Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. Embo J *18*, 5205-5215.

Murakami, S., Lefebvre, V., and de Crombrugghe, B. (2000). Potent inhibition of the master chondrogenic factor sox9 gene by interleukin-1 and tumor necrosis factor-alpha [In Process Citation]. J Biol Chem *275*, 3687-3692.

Nagase, H. (1997). Activation mechanisms of matrix metalloproteinases. Biol Chem *378*, 151-160.

Nagase, H. (1998). Stromelysins 1 and 2. In Matrix Metalloproteinases, W. C. Parks, and R. P. Mecham, eds. (San Diego, CA, Academic Press), pp. 43-84.

Nagase, H., and Woessner, J. F., Jr. (1999). Matrix metalloproteinases. J Biol Chem *274*, 21491-21494.

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., and Sugino, H. (1990). Activin-binding protein from rat ovary is follistatin. Science *247*, 836-838.

Nakanishi, T., Kimura, Y., Tamura, T., Ichikawa, H., Yamaai, Y., Sugimoto, T., and Takigawa, M. (1997). Cloning of a mRNA preferentially expressed in chondrocytes by differential display-PCR from a human chondrocytic cell line that is identical with connective tissue growth factor (CTGF) mRNA. Biochem Biophys Res Commun *234*, 206-210.

Nakanishi, T., Nishida, T., Shimo, T., Kobayashi, K., Kubo, T., Tamatani, T., Tezuka, K., and Takigawa, M. (2000). Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. Endocrinology *141*, 264-273.

Neidel, J. (2001). Changes in systemic levels of insulin-like growth factors and their binding proteins in patients with rheumatoid arthritis. Clin Exp Rheumatol *19*, 81-84.

Neumann, E., Barnum, S. R., Tarner, I. H., Echols, J., Fleck, M., Judex, M., Kullmann, F., Mountz, J. D., Scholmerich, J., Gay, S., and Muller-Ladner, U. (2002). Local production of complement proteins in rheumatoid arthritis synovium. Arthritis Rheum *46*, 934-945.

Nguyen, M., Arkell, J., and Jackson, C. J. (2000). Activated protein C directly activates human endothelial gelatinase A. J Biol Chem *275*, 9095-9098.

Nyirkos, P., and Golds, E. E. (1990). Human synovial cells secrete a 39 kDa protein similar to a bovine mammary protein expressed during the non-lactating period. Biochem J *269*, 265-268.

Oegema, T., and Thompson, R. (1992). The zone of calcified cartilage. In Articular Cartilage and Osteoarthritis, K. E. Kuettner, R. Schleyerbach, J. G. Peyron, and V. C. Hascall, eds. (New York, Raven Press), pp. 319-331.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. J Biol Chem *250*, 4007-4021.

Ollivierre, F., Gubler, U., Towle, C. A., Laurencin, C., and Treadwell, B. V. (1986). Expression of IL-1 genes in human and bovine chondrocytes: a mechanism for autocrine control of cartilage matrix degradation. Biochem Biophys Res Commun *141*, 904-911.

Pangas, S. A., and Woodruff, T. K. (2000). Activin signal transduction pathways. Trends Endocrinol Metab *11*, 309-314.

Pavloff, N., Staskus, P. W., Kishnani, N. S., and Hawkes, S. P. (1992). A new inhibitor of metalloproteinases from chicken: ChIMP-3. A third member of the TIMP family. J Biol Chem *267*, 17321-17326.

Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis *20*, 3551-3567.

Pfander, D., Kortje, D., Zimmermann, R., Weseloh, G., Kirsch, T., Gesslein, M., Cramer, T., and Swoboda, B. (2001). Vascular endothelial growth factor in articular cartilage of healthy and osteoarthritic human knee joints. Ann Rheum Dis *60*, 1070-1073.

Poole, A. R. (1986). Proteoglycans in health and disease: structures and functions. Biochem J *236*, 1-14.

Pujol, J. P., Galera, P., Pronost, S., Boumediene, K., Vivien, D., Macro, M., Min, W., Redini, F., Penfornis, H., Daireaux, M., and et al. (1994). Transforming growth factor-beta (TGF-beta) and articular chondrocytes. Ann Endocrinol (Paris) *55*, 109-120.

Pullig, O., Weseloh, G., and Swoboda, B. (1999). Expression of type VI collagen in normal and osteoarthritic human cartilage. Osteoarthritis Cartilage *7*, 191-202.

Pullig, O., Weseloh, G., Gauer, S., and Swoboda, B. (2000). Osteopontin is expressed by adult human osteoarthritic chondrocytes: protein and mRNA analysis of normal and osteoarthritic cartilage. Matrix Biol *19*, 245-255.

Pullig, O., Weseloh, G., Klatt, A. R., Wagener, R., and Swoboda, B. (2002). Matrilin-3 in human articular cartilage: increased expression in osteoarthritis. Osteoarthritis Cartilage *10*, 253-263.

Reddi, A. H. (1992). Regulation of cartilage and bone differentiation by bone morphogenetic proteins. Curr Opin Cell Biol *4*, 850-855.

Redini, F., Galera, P., Mauviel, A., Loyau, G., and Pujol, J. P. (1988). Transforming growth factor beta stimulates collagen and glycosaminoglycan biosynthesis in cultured rabbit articular chondrocytes. FEBS Lett *234*, 172-176.

Redini, F., Mauviel, A., Pronost, S., Loyau, G., and Pujol, J. P. (1993). Transforming growth factor beta exerts opposite effects from interleukin-1 beta on cultured rabbit articular chondrocytes through reduction of interleukin-1 receptor expression. Arthritis Rheum *36*, 44-50.

Roepstorff, P., and Fohlman, J. (1984). Proposal for a common nomenclature for sequence ions in mass spectra of peptides [letter]. Biomed Mass Spectrom *11*, 601.

Rosenberg, K., Olsson, H., Morgelin, M., and Heinegard, D. (1998). Cartilage oligomeric matrix protein shows high affinity zinc-dependent interaction with triple helical collagen. J Biol Chem *273*, 20397-20403.

Roughley, P. J., White, R. J., Cs-Szabo, G., and Mort, J. S. (1996). Changes with age in the structure of fibromodulin in human articular cartilage. Osteoarthritis Cartilage *4*, 153-161.

Sah, R. L., Chen, A. C., Grodzinsky, A. J., and Trippel, S. B. (1994). Differential effects of bFGF and IGF-I on matrix metabolism in calf and adult bovine cartilage explants. Arch Biochem Biophys *308*, 137-147.

Sakai, R., Miwa, K., and Eto, Y. (1999). Local administration of activin promotes fracture healing in the rat fibula fracture model. Bone *25*, 191-196.

Saklatvala, J. (1987). Interleukin 1: purification and biochemical aspects of its action on cartilage. J Rheumatol *14 Spec No*, 52-54.

Saklatvala, J., Davis, W., and Guesdon, F. (1996). Interleukin 1 (IL1) and tumour necrosis factor (TNF) signal transduction. [Review] [40 refs]. Philos Trans R Soc Lond B Biol Sci *351*, 151-157.

Sandy, J. D., Neame, P. J., Boynton, R. E., and Flannery, C. R. (1991). Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. J Biol Chem *266*, 8683-8685.

Sato, H., Takino, T., Kinoshita, T., Imai, K., Okada, Y., Stetler Stevenson, W. G., and Seiki, M. (1996). Cell surface binding and activation of gelatinase A induced by expression of membrane-type-1-matrix metalloproteinase (MT1-MMP). FEBS Lett *385*, 238-240.

Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994). A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature *370*, 61-65.

Schalkwijk, J., Joosten, L. A., van den Berg, W. B., van Wyk, J. J., and van de Putte, L. B. (1989). Insulin-like growth factor stimulation of chondrocyte proteoglycan synthesis by human synovial fluid. Arthritis Rheum *32*, 66-71.

Schubert, D., Kimura, H., LaCorbiere, M., Vaughan, J., Karr, D., and Fischer, W. H. (1990). Activin is a nerve cell survival molecule. Nature *344*, 868-870.

Schubert, M., and Hamerman, D. (1968). A primer on conncetive tissue biochemistry (Philadelphia, Lea & Febiger).

Scott, J. E., and Haigh, M. (1988). Identification of specific binding sites for keratan sulphate proteoglycans and chondroitin-dermatan sulphate proteoglycans on collagen fibrils in cornea by the use of cupromeronic blue in 'critical-electrolyte-concentration' techniques. Biochem J *253*, 607-610.

Serra, R., Johnson, M., Filvaroff, E. H., LaBorde, J., Sheehan, D. M., Derynck, R., and Moses, H. L. (1997). Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J Cell Biol *139*, 541-552.

Shackelton, L. M., Mann, D. M., and Millis, A. J. (1995). Identification of a 38-kDa heparin-binding glycoprotein (gp38k) in differentiating vascular smooth muscle cells as a member of a group of proteins associated with tissue remodeling. J Biol Chem *270*, 13076-13083.

Shakibaei, M., De Souza, P., and Merker, H. J. (1997). Integrin expression and collagen type II implicated in maintenance of chondrocyte shape in monolayer culture: an immunomorphological study. Cell Biol Int *21*, 115-125.

Shen, Z., Heinegard, D., and Sommarin, Y. (1995). Distribution and expression of cartilage oligomeric matrix protein and bone sialoprotein show marked changes during rat femoral head development. Matrix Biol *14*, 773-781.

Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Boucherie, H., and Mann, M. (1996a). Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proc Natl Acad Sci U S A *93*, 14440-14445.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996b). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem *68*, 850-858.

Silver, I. A. (1975). Measurement of pH and ionic composition of pericellular sites. Philos Trans R Soc Lond B Biol Sci *271*, 261-272.

Sinz, A., Bantscheff, M., Mikkat, S., Ringel, B., Drynda, S., Kekow, J., Thiesen, H. J., and Glocker, M. O. (2002). Mass spectrometric proteome analyses of synovial fluids and plasmas from patients suffering from rheumatoid arthritis and comparison to reactive arthritis or osteoarthritis. Electrophoresis *23*, 3445-3456.

Sipe, J. D. (1995). Acute-phase proteins in osteoarthritis. Semin Arthritis Rheum *25*, 75-86.

Smith, J. C. (1993). Mesoderm-inducing factors in early vertebrate development. Embo J *12*, 4463-4470.

Soder, S., Hambach, L., Lissner, R., Kirchner, T., and Aigner, T. (2002). Ultrastructural localization of type VI collagen in normal adult and osteoarthritic human articular cartilage. Osteoarthritis Cartilage *10*, 464-470.

Song, J. J., Celeste, A. J., Kong, F. M., Jirtle, R. L., Rosen, V., and Thies, R. S. (1995). Bone morphogenetic protein-9 binds to liver cells and stimulates proliferation. Endocrinology *136*, 4293-4297.

Steck, E., Benz, K., Lorenz, H., Loew, M., Gress, T., and Richter, W. (2001). Chondrocyte expressed protein-68 (CEP-68), a novel human marker gene for cultured chondrocytes. Biochem J *353*, 169-174.

Stokes, D. G., Liu, G., Coimbra, I. B., Piera-Velazquez, S., Crowl, R. M., and Jimenez, S. A. (2002). Assessment of the gene expression profile of differentiated and dedifferentiated human fetal chondrocytes by microarray analysis. Arthritis Rheum *46*, 404-419.

SundarRaj, N., Fite, D., Ledbetter, S., Chakravarti, S., and Hassell, J. R. (1995). Perlecan is a component of cartilage matrix and promotes chondrocyte attachment. J Cell Sci *108 (Pt 7)*, 2663-2672.

Suzuki, T., Bessho, K., Fujimura, K., Okubo, Y., Segami, N., and Iizuka, T. (2002). Regeneration of defects in the articular cartilage in rabbit temporomandibular joints by bone morphogenetic protein-2. Br J Oral Maxillofac Surg *40*, 201-206.

Tetlow, L. C., Adlam, D. J., and Woolley, D. E. (2001). Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum *44*, 585-594.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W., and Melton, D. A. (1990). Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. Cell *63*, 485-493.

Timpl, R., and Engel, J. (1987). Type VI collagen. In: Mayne, R. and Burgeson, R., eds. Structure and function of collagen types. (London: Academic press inc.), pp. 105-143.

Tortora, and Grabowski (1994). Principles of anatomy and physiology, 7 edn, Harper Collins).

Tortorella, M. D., Burn, T. C., Pratta, M. A., Abbaszade, I., Hollis, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wynn, R., *et al.* (1999). Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins [see comments]. Science *284*, 1664-1666.

Towle, C. A., Hung, H. H., Bonassar, L. J., Treadwell, B. V., and Mangham, D. C. (1997). Detection of interleukin-1 in the cartilage of patients with osteoarthritis: a possible autocrine/paracrine role in pathogenesis. Osteoarthritis Cartilage *5*, 293-300.

Tyler, J. (1991). Cartilage degradation. In Cartilage:Molecular Aspects, B. Hall, and S. Newman, eds. (Boca raton, FL, CRC Press), pp. 213-256.

Urban, J. P. (1994). The chondrocyte: a cell under pressure. Br J Rheumatol *33*, 901-908.

Vallon, R., Freuler, F., Desta-Tsedu, N., Robeva, A., Dawson, J., Wenner, P., Engelhardt, P., Boes, L., Schnyder, J., Tschopp, C., *et al.* (2001). Serum amyloid A (apoSAA) expression is up-regulated in rheumatoid arthritis and induces transcription of matrix metalloproteinases. J Immunol *166*, 2801-2807.

Verheijden, G. F., Rijnders, A. W., Bos, E., Coenen-de Roo, C. J., van Staveren, C. J., Miltenburg, A. M., Meijerink, J. H., Elewaut, D., de Keyser, F., Veys, E., and Boots, A. M. (1997). Human cartilage glycoprotein-39 as a candidate autoantigen in rheumatoid arthritis. Arthritis Rheum *40*, 1115-1125.

Viard, I., Wehrli, P., Jornot, L., Bullani, R., Vechietti, J. L., Schifferli, J. A., Tschopp, J., and French, L. E. (1999). Clusterin gene expression mediates resistance to apoptotic cell death induced by heat shock and oxidative stress. J Invest Dermatol *112*, 290-296.

Villiger, P. M., and Lotz, M. (1992). Differential expression of TGF beta isoforms by human articular chondrocytes in response to growth factors. J Cell Physiol *151*, 318-325.

Vincent, T., Hermansson, M., Bolton, M., Wait, R., and Saklatvala, J. (2002). Basic FGF mediates an immediate response of articular cartilage to mechanical injury. Proc Natl Acad Sci U S A *99*, 8259-8264.

Volck, B., Price, P. A., Johansen, J. S., Sorensen, O., Benfield, T. L., Nielsen, H. J., Calafat, J., and Borregaard, N. (1998). YKL-40, a mammalian member of the chitinase family, is a matrix protein of specific granules in human neutrophils. Proc Assoc Am Physicians *110*, 351-360.

Vorm, O., Roepstorff, P., and Mann, M. (1994). Improved resolution and very high sensitivity in MALDI TOF of matrix surfaces made by fast evaporation. Anal Chem *66*, 3281-3287.

Wait, R. (1993). Introduction to mass spectrometry. In Spectroscopic methods and analyses, C. Jones, B. Mulloy, and A. H. Thomas, eds. (Totowa, New Jersey, Humana Press Inc), pp. 192.

Wait, R., Gianazza, E., Eberini, I., Sironi, L., Dunn, M. J., Gemeiner, M., and Miller, I. (2001). Proteins of rat serum, urine, and cerebrospinal fluid: VI. Further protein identifications and interstrain comparison. Electrophoresis *22*, 3043-3052.

Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M. W., Harris, R., Williams, K. L., and Humphery-Smith, I. (1995). Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium. Electrophoresis *16*, 1090-1094.

Weckmann, A. L., and Alcocer-Varela, J. (1996). Cytokine inhibitors in autoimmune disease. [Review] [82 refs]. Semin Arthritis Rheum *26*, 539-557.

Weekes, J., Wheeler, C. H., Yan, J. X., Weil, J., Eschenhagen, T., Scholtysik, G., and Dunn, M. J. (1999). Bovine dilated cardiomyopathy: proteomic analysis of an animal model of human dilated cardiomyopathy. Electrophoresis *20*, 898-906.

Wiley, W. C., and McLaren, I. H. (1955). Time-of-flight mass spectrometer with improved resolution. Rev Sci Instr *26*, 1150-1157.

Wilkins, M. R., Hochstrasser, D. F., Sanchez, J. C., Bairoch, A., and Appel, R. D. (1996). Integrating two-dimensional gel databases using the Melanie II software. Trends Biochem Sci *21*, 496-497.

Wilm, M., and Mann, M. (1996). Analytical properties of the nanoelectrospray ion source. Anal Chem *68*, 1-8.

Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Fotsis, T., and Mann, M. (1996). Femtomole sequencing of proteins from polyacrylamide gels by nano- electrospray mass spectrometry. Nature *379*, 466-469.

Wisniewski, H. G., Maier, R., Lotz, M., Lee, S., Klampfer, L., Lee, T. H., and Vilcek, J. (1993). TSG-6: a TNF-, IL-1-, and LPS-inducible secreted glycoprotein associated with arthritis. J Immunol *151*, 6593-6601.

Woessner, J. F., Jr. (1998). The matrix metalloproteinase family. In Matrix Metalloproteinases, W. C. Parks, and R. P. Mecham, eds. (San Diego, CA, Academic Press), pp. 1-14.

Wong, G. H., and Goeddel, D. V. (1988). Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. Science *242*, 941-944.

Wong, G. H. (1995). Protective roles of cytokines against radiation: induction of mitochondrial MnSOD. [Review] [15 refs]. Biochim Biophys Acta *1271*, 205-209.

Wong, M., Wuethrich, P., Eggli, P., and Hunziker, E. B. (1996). Zone-specific cell biosynthetic activity in mature bovine articular cartilage: a new method using confocal microscopic sterelogy and quantitative autoradiography. J Ortho Res *14*, 424-432.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994). Mechanism of activation of the TGF-beta receptor. Nature *370*, 341-347.

Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. (1968). Purification and properties of bacterial chondroitinases and chondrosulfatases. J Biol Chem *243*, 1523-1535.

Yamaguchi, Y., Mann, D. M., and Ruoslahti, E. (1990). Negative regulation of transforming growth factor-beta by the proteoglycan decorin. Nature *346*, 281-284.

Yates, J. R., 3rd, Eng, J. K., McCormack, A. L., and Schieltz, D. (1995a). Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. Anal Chem *67*, 1426-1436.

Yates, J. R., 3rd, Eng, J. K., and McCormack, A. L. (1995b). Mining genomes: correlating tandem mass spectra of modified and unmodified peptides to sequences in nucleotide databases. Anal Chem *67*, 3202-3210.

Yates, J. R., 3rd, Carmack, E., Hays, L., Link, A. J., and Eng, J. K. (1999). Automated protein identification using microcolumn liquid chromatography-tandem mass spectrometry. Methods Mol Biol *112*, 553-569.

Yoshihara, Y., Nakamura, H., Obata, K., Yamada, H., Hayakawa, T., Fujikawa, K., and Okada, Y. (2000). Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. Ann Rheum Dis *59*, 455-461.

Appendix 1

Identification of Proteins from Porcine Explant Medium by Mass Spectrometry

Proteins from the gel in fig 4.13 were identified by MALDI and ESI MS/MS

Spot	Protein	Exp pI/Mw	Theor. pI/Mw	Sequence
A1	Connective tissue growth factor (CTGF)	6.8/21000	8.38/35404	QLGEL CTER LPSPD CPFPR GLFCD FGSPA NRK CPAGV SLVLD GCGCC R QLGEL CTERD PCDPH K
A2	Peptidyl-prolyl cis-trans isomerase B	10/20000	9.13/20158	IGDED IGR DVTIA DCGK TVDNF VALAT GEK DTNGS QFFIT TVK SIYGE RFPDE NFK VIKDF MIQGG DFTR
	Cartilage oligomeric matrix protein (COMP)	10/20000	4.35/80934	SSTGP GEQLR NALWH TGDTA SQVR
A3	Tetranectin	5.9/21000	5.8/20168	EQQAL QTVCL K TFHEA SEDCI SR NWETE ITAQP DGGK
	Connective tissue growth factor (CTGF)	5.9/21000	8.38/35404	SGESF QSSSCK DGAPC VFGGT VYR
A4	Metalloproteinase inhibitor 1 (TIMP-1)	8.8/27000	8.29/20690	GFNAL GDAPD IR EPGMC TWQSL RPR FIYTP AMESV CGYFH R LQSDT HCLWT DQLLT GSDK
A5	Metalloproteinase inhibitor I (TIMP-1)	8.5/27000	8.29/20690	GFNAL GDAPD IR
	Immunoglobulin	8.5/27000	9.01/11519	LLIYA ASSLQ SGVPS R
A6	Triosephosphate isomerase	7.3/28000	6.51/26538	IAVAA QNCYK IIYGG SVTGA TCK HVFGE SDELI GQK TATPQ QAQEV HEK VVLAY EPVWA IGTGK VAHAL AEGLG VIACI GEK ELASQ PDVDG FLVGG ASLKP EFVDI INAK
	Collagen alpha 1 type II C-propeptide	7.3/28000	6.66/27405	ALLIQ GSNDV EIR NSIAY LDEAA GNLKK
	Haemoglobin beta chain	7.3/28000	7.25/16034	LLGNV LVVVL AR
A7	Triosephosphate isomerase	7.4/28000	6.51/26538	FFVGG NWK IAVAA QNCYK IIYGG SVTGA TCK HVFGE SDELI GQK TATPQ QAQEV HEK VVLAY EPVWA IGTGK VAHAL AEGLG VIACI GEK ELASQ PDVDG FLVGG ASLKP EFVDI INAK
A8	Phosphoglycerate mutase	6.6/28000	6.75/28672	HYGGL TGLNK HGESSA WNLEN R FSGWY DADLS PAGHE EAK ALPFW NEEIV PQIKE GK YADLT EDQLP SCESL KDTIA R
	Triosephosphate isomerase	6.6/28000	6.51/26538	IAVAA QNCYK IIYGG SVTGA TCK TATPQ QAQEV HEK
A9	Collagen alpha 1 type II C-propeptide	6.6/32000	6.66/27405	KNWWS SK LCHPE WK SLNNQ IESIR ALLIQ GSNDV EIR NSIAY LDEAA GNLKK SGDYW IDPNQ GCTLD AMK
A10	Collagen alpha 1 type II C-propeptide	7.2/36000	6.66/27405	KNWWS SK NSIAY LDEAA GNLKK ALLIQ GSNDV EIRAE GNSR

A11	Collagen alpha 1 type II C-propeptide	6.5/36000	6.66/27405	KNWWS SK NSIAY LDEAA GNLKK ALLIQ GSNDV EIRAE GNSR
A12	Collagen alpha 1 type II C-propeptide	6.2/36000	6.66/27405	KNWWS SK WGKTM IEYR KALLI QGSND VEIR NSIAY LDEAA GNLKK ALLIQ GSNDV EIRAE GNSR HIWFGETINGGFHFSYGDDNLAPNTADVQMTFLR
	Malate dehydrogenase	6.2/36000	6.15/36323	VLVTG AAGQI AYSLL YSIGN GSVFG K
	Serum albumin	6.2/36000	5.92/69410	TVLGN FAAFV QK LSCAE DYLSL VLNR
B1	Collagen alpha 1 type II C-propeptide	5.8/36000	6.66/27405	FTYTV LKDGC TK NSIAY LDEAA GNLKK ALLIQ GSNDV EIRAE GNSR
B2	L-lactate dehydrogenase B chain	5.4/37000	5.58/36481	GLTSV INQK LKDDE VAQLK NSADT LWGIQ K VIGSG CNLDS AR MVVES AYEVI K SLTDE LALVD VLEDK
	Collagen alpha 1 type II C-propeptide	5.4/37000	6.66/27405	ALLIQ GSNDV EIR NSIAY LDEAA GNLKK
B3	Collagen alpha 1 type II C-propeptide	5.3/37000	6.66/27405	FTYTV LEDGC TK SGEYW VDPNQ GCK SVNGQ IESLI SPDGS R ALLIQ GSNDV EIR
	Thrombospondin 1	5.3/37000	4.74/129553	AQLYI DCEK SITLF VQEDR FVFGT TPEDI LR
	Collagen alpha 1 (XI) chain	5.3/37000	5.08/181120	QAQLS APTK TTGFC TNR AIVDT NGITV FGTR
B4	Collagen alpha 1 (XI) chain	4/38000	5.08/181120	AIVDT NGITV FGTR
B5	Collagen alpha 1 (XI) chain	4.2/38000	5.08/181120	ALYDG CASR DLQLS HPDFP DGEYW IDPNQ GCSGD SFK
B6	Annexin A8	5/36000	5.56/36879	VFEEY EK IRGTD EMK ILVCL LQGSR EGVII EILAS R GTDEM KFITI DVLTK GIGTN EQAII DVLTK R SIEDS IKSET HGSLE EAMLT VVK
	Thrombospondin 1	5/36000	4.74/129553	GPDPS SPAFR SITLF VQEDR
	Collagen alpha 1 (XI) chain	5/36000	5.08/181120	VAIVSV EKK AIVDT NGITV FGTR
B7	Collagen alpha 1 (XI) chain	7.5/26000	5.08/181120	VAISV EKK QAQLS APTK TTGFC TNRK TVNIA DGKWH R ALDFH NSPEG ISK AIVDT NGITV FGTR
B8	Angiopoietin-related protein 2	8/26000	7.07/54802	DNALE LSQLE NR VTQLY MQLLH EIIR VTGAI CVNSK EPEVL LENR
	Glutathione S-transferase P	8/26000	8.07/23496	PPYTI TYFPV R IHQVL NPSCL DAFPL LSAYV AR
	Collagen alpha 1 (XI) chain	8/26000	5.08/181120	TTGFC TNR AIVDT NGITV FGTR
B9	Osteronectin/SPARC	4.2/40000	4.66/32697	NVLVT LYER LHLDY IGPCK TFDSS CHFFA TK RLEAG DHPVE LLAR

				YIALD EWAGC FGIK YIPPC LDSEL TEFPL R FFETC DLDND KYIAL DEWAG CFGIK
	Clusterin	4.2/40000	5.7/48695	EPFDT QFFSP FGSSH R
	Heat shock 70 kDa protein	4.2/40000	5.32/69199	LLQDF FNGK
B10	Osteronectin/SPARC	4.4/39000	4.66/32697	LEAGD HPVEL LAR TFDSS CHFFA TK
B11	Osteronectin/SPARC	4.6/38000	4.66/32697	NVLVT LYER LEAGD HPVEL LAR
B12	Osteronectin/SPARC	4.1/41000	4.66/32697	NVLVT LYER LHLDY IGPCK TFDSS CHFFA TK YIPPC LDSEL TEFPL R
C1	Osteronectin/SPARC	4.1/42000	4.66/32697	NVLVT LYER LHLDY IGPCK TFDSS CHFFA TK YIPPC LDSEL TEFPL R EFETC DLDND KYIAL DEWAG CFGIK
C2	Aspartate aminotransferase	9.7/43000	8.98/44664	IGANF LQR VGAFT VVCK IASTI LTSPD LR ASAEL ALGEN NEVLK
C3	Matrix metalloproteinase-1 (MMP-1)	5.6/59000	5.41/511644	LTFDA ITTLR NFRDY NLYR DGFLY FFHGT R NIDAA VFEED TGK TYFFV AHECW R NYYNL NSDGV PVEK YDEYK QSMDT GYPK AFQLW SNVSP LTFTK IENYT PDLSR EDVDR
C4	Polyubiquitin	5.4/14000	7.13/68497	EGIPP DQQR ESTLH LVLR TLSDY NIQK IQDKE GIPPD QQR TITLE VEPSD TIENV KAK
C5	Polyubiquitin	5.6/14000	7.13/68497	IQDKE GIPPD QQR
C6	Calpactin 1 light chain	6.3/14000	6.35/10943	FAGDK GYLTK EFPGF LENQK DPLAV DK
C7	Elongation factor 1-delta	6.7/14000	4.9/31121	IASLE VENQS LR
C8	Haemoglobin beta chain	7.4/14000	7.25/16034	VLQSF SDGLK VVAGV ANALA HK KVLQS FSDGL K LLGNV IVVVL AR LLVVY PWTQR VNVDE VGGEA LGR LGHDF NPNVQ AAFQK VHLSA EEKEA VLGLW GK FFESF GDLSN ADAVM GNPK LSELH CDQLH VDPEN FR
C9	Haemoglobin beta chain	8/14000	7.25/16034	EAVLG LWGK VLQSF SDGLK VVAGV ANALA HK LLGNV IVVVL AR LLVVY PWTQR VNVDE VGGEA LGR RLGHD FNPNV QAAFQ K VHLSA EEKEA VLGLW GK FFESF GDLSN ADAVM GNPK LSELH CDQLH VDPEN FR
	Haemoglobin epsilon chain	8/14000	8.73/15963	LLVVY PWYQR LSELH CDKLH VDPEN FR
C10	Haemoglobin beta chain	7.7/14000	7.25/16034	VLQSF SDGLK LLGNV IVVVL AR

				VNVDE VGGEA LGR LGHDF NPNVQ AAFQK
C11	Superoxide dismuatse [Cu-Zn]	6.5/18000	6.04/15760	TVLVT GTIK HVGDL GNVTA GK
	Haemoglobin beta chain	6.5/18000	7.25/16034	LLGNV LVVVL AR
C12	Haemoglobin beta chain	6.8/19000	7.25/16034	LLGNV LVVVL AR
D1	Fibrogen A-alpha-chain	8.3/18000	6.55/47354	DQYLPL IK VDLKD YENQQ K QLKQV IAINL LPSRD R
	Nucloeside diphosphate kinase B	8.3/18000	8.52/17298	GLVGE IIKR GDFCI QVGR NIIHG SDSVK
D2	Cofilin, non-muscle isoform	8.5/19000	8.16/18518	MLPDK DCR AVLFC LSEDK K YALYD ATYET K HELQA NCYEE VKDR NIILE EGKEI LVGVD GQTVD DPYAT FVK
D3	Phosphatidylethanolamine-binding protein	8.2/21000	7.39/20854	VLTPT QVK CDEPI LSNR YGGAE VDELG K GNNIS SGTVL SDYVG SGPPK GTGLH R
D4	Peroxiredoxin 1	7.5/21000	8.26/22176	QITIN DLPVG R GLFII DDKGI LR TIAQD YGVLK ADEGI SFR
	Peroxiredoxin 4	7.5/21000	5.86/30539	QITLN DLPVG R GLFII DDKGI LR
D5	Peroxiredoxin 1	8.1/22000	8.26/22176	LVQAF QFTDK GLFII DDKGI LR TIAQD YGVLK ADEGI SFR
	Peroxiredoxin 4	8.1/22000	5.86/30539	QITLN DLPVH R GLFII DDKGI LR
D6	Peroxiredoxin 1	8.9/24000	8.26/22176	TIAQD YGVLK QITIN DLPVG
	Peroxiredoxin 4	8.9/24000	5.86/30539	QITLN DLPVG R
D7	Peroxiredoxin 1	9.1/24000	8.26/22176	QITIN DLPVG R GLFII DDKGI LR TIAQD YGVLK ADEGI SFR
	Ig lambda chain C region	9.1/24000	6.76/11003	AGGTT VTQGV ETTKP SK AAPTV NLFPP SSEEL GTNK
	Peroxiredoxin 4	9.1/24000	5.86/30539	QITLN DLPVG R GLFII DDKGI LR
D8	DJ-1 protein	5.8/23000	6.33/19847	DGLIL TSR RALVI LAK VTVAG LAGKD PVQCS GAEEM ETVIP VDVMR EGPYD VVVLP GGNLG AQNLS ESAAV K
D9	RNA-binding protein regulatory subunit	6.4/23000	6.33/19891	DGLIL TSR RALVI LAK VTVAG LAGKD PVQCS R GAEEM ETVIP VDVMR GLIAA ICAGP TALLA HEIGF GSK
D10	Ig lambda chain C region	5.9/24000	6.76/11003	AGGTT VTQGV ETTKP SK SSSGF TCQVT HEGTI VEK AAPTV NLFPP SSEEL GTNK QSNNK YAASS YLALS ASDWK
	Angiopoietin 1 receptor	5.9/24000	7.22/124183	KLLYK PVNHY EAWR
	Thioredoxin-dependent peroxide reductase	5.9/24000	5.73/21529	GLFII DPNGV IKHLS VNDLP VGR
D11	Ig lambda chain C region	6.9/24000	6.76/11003	YAASS YLALS ASDWK AGGTT VTQGV ETTKP SK SSSGF TCQVT HEGTI VEK AAPTV NLFPP SSEEL GTNK
D12	Ig lambda chain C region	7.8/24000	6.76/11003	SSSGF TCQVT HEGTI VEK
E1	Superoxide dismutase [Mn], mitochondrial	8/24000	6.86/22204	GELLE AIKR

				GDVTA QVALQ PALK
E2	Glutathione S-transferase P	8.5/25000	8.07/23496	PPYTI TYFPV R
				YATLI YTNYE AGKEK
				EAALV DMVND GVEDL R
				FQDGD LTLYQ SNAIL R
				AFLAS PEHVN RPING NGK
				IHQVL NPSCL DAFPL LASYV AR
				MLLAD QDQSW KEEVV TMETW PPLKP SCLFR
E3	Thrombospondin 1	6.3/28000	4.74/129553	GPDPS SPAFR
				AQLYI DCEK
				SITLF VQEDR
				AGTLD LSLTV QGK
				FVFGT TPEDI LR
				GGVND NFQGV LQNVR
				IPESG GDNSV FDIFE LTGAA R
E4	Thrombospondin 1	5.5/28000	4.74/129553	GPDPS SPAFR
				AQLYI DCEK
				SITLF VQEDR
				AEKGF LLLAS LR
				FVFGT TPEDI LR
				GGVND NFQGV LQNVR
E5	Thrombospondin 1	5.5/29000	4.74/129553	GPDPS SPAFR
				GGVND NFQGV LQNVR
E6	Thrombospondin 1	5.2/35000	4.74/129553	GPDPS SPAFR
				TRGTL LAVER
				AQLYI DCEK
				SITLF VQEDR
				AGTLD LSLTV QGK
				AEKGF LLLAS LR
				GGVND NFQGV LQNVR
				MENAE LDVPI QSIFT R
				IPESG GDNSV FDIFE LTGAA R
E7	Thrombospondin-1	5.3/39000	4.74/129553	FVFGT TPEDI LR
				NRIPE SGGDN SVFDI FELTG AAR
	Inorganic pyrophosphatase	5.3/39000	5.27/32844	YVANL FPYK
				LKPGY LEATV DWFRR
E8	Collagen alpha 1 type II C-propeptide	5.4/36000	6.66/27405	NSIAY LDEAA GNLKK
				ALLIQ GSNDV EIRAE GNSR
	F-actin capping protein alpha-2 subunit	5.4/36000	4.73/13589	LLLNN DNLLR
				FIIHA PPGEF NEVFN DVR
	Thrombospondin 1	5.4/36000	4.74/129553	FVFGT TPEDI LR
				IEDAN LIPPV PDDKF QDLVD AVR
E9	Phosphoglycerate mutase	5.9/30000	6.75/28672	VLIAA HGNSL
				RVLIA AHGNS LR
				HGESA WNLEN R
				HYGGL TGLNK AETAA K
				ALPFW NEEIV PQIK
				DAGYE FDICF TSVQK R
				FSGWY DADLS PAGHE EAKR
				YADLT EDQLP SCESL KDTIA R
	Non-selenium glutathione phospholipid hydroxyperoxide peroxidase	5.9/30000	5.73/25036	DFTPV CTTEL GR
				LKLSI LYPAT TGR
				ETLPF PIIDD KSR
				FHDFL GDSWG ILFSH PR
				DINAY NGEEP KETLP FPIID DK
E10	Phosphoglycerate mutase	6.3/31000	6.75/28672	HYGGL TGLNK
				HGESA WNLEN R
				ALPFW NEEIV PQIKE GK
				FSGWY DADLS PAGHE EAKR
				YADLT EDQLP SCESL KDTIA R
E11	Collagen alpha 1 type II C-propeptide	6.4/32000	6.66/27405	KNWWS SK
				ALLIQ GSNDV EIR
				NSIAY IDEAA GNLKK
				SLNNQ IESIR SPEGS R

E12	Collagen alpha 1 type II C-propeptide	5.8/32000	6.66/27405	SLNNQ IESIR
F1	Collagen alpha 1 type II C-propeptide	7.6/34000	6.66/27405	DLKLC HPEWK ALLIQ GSNDV EIR NSAIY LDEAA GNLKK SLNNQ IESIR SPEGS R
	Type IX collagen alpha 1 chain	7.6/34000	8.69/20906	IGQDD LPGFD LISQF QIDK
F2	L-lactate dehydrogenase A chain	8.6/35000	8.22/36487	DQLIH NLLK SADTL WGIQK VIGSG CNLDS AR VTLTP EEEAH LKK IVSGK DYNVT ANSR ELADE IALVD WMEDK LLVVS NPVDI LTYVA WK GYTSW AIGLS VADLA ESIMK GLYGI KEDVF LSVPC ILGQN GISDV VK
F3	60S acidic ribosomal protein P0	9.6/34000	5.55/33257	AGAIA PCEVT VPAQN TGLGP EK
F4	Chondroadherin	9.8/34000	9.43/38443	AMPNL VSLHL QHCQI R GLLSP LVNLF ILQLN NNK FYLDR NQLSS YPSAA LSK FSDGA FLGVT TLKHV HLENN R AGAFD DLTEL TYLYL DHNKV TELPR
F5	Fructose-bisphosphate aldolase A	9.3/39000	8.39/39288	CQYVT EK AAQEE YVKR ALANS LACQG K GILAA DESTG SIAKR CPLLK PWALT FSYGR VNPCI GGVIL FHETL YQK GVVPL AGTNG ETTTQ GLDGL SER VDKGV VPLAG TNGET TTQGL DGLSE R YASIC QQNGI VPIVE PEILP DGDHD LKR
F6	Procollagen C-proteinase enhancer protein	5/39000	7.55/45549	FCGTF RPAPL VAPGN QVTLR
	Annexin A8	5/39000	5.56/36879	GIGTN EQAII DVLTK R
F7	Alpha-1 acid glycoprotein	3/50000	5.83/20898	DLCGP LEK KDLCG PLEK SEITY TDEKK EHVAD LLLTK VPK EFHDA IECTG IHK SIQAA FFFFD PKFFD PKPAE DKINL R
F8	Actin beta chain	5.2/48000	5.29/41605	SYELP DGQVI TIGNE R VAPEE HPVIL TEAPL NPK DLYAN TVLSG GTTMY PGIAD R LCYVA LDFEQ EMATA ASSSS LEK TTGIV MDSGD GVTHT VPIYE GYALP HAILR
F9	Alpha enolase	5.4/41000	6.99/47037	YISPD QLADL YK AAVPS GASTG IYEAL ELR
F10	Fibrinogen beta chain	5.4/45000	7.15/52314	ECEEI IR YCGLP GEYWL GNDKI SQLTR
F11	Fibrinogen beta chain	5.6/45000	7.15/52314	GGWTV IQNR EDGGG WWYNR TPCTV TCNIP VVSGK
F12	Fibrinogen beta chain	6/45000	7.15/52314	ECEEI IR EDGGG WWYNR DNENV VNEYS SELEK HQLYI DETVNSNIPTNLR
G1	Ornithine aminotransferase, mitochondrial	6.3/50000	6.57/48534	DNGLL AKPTH GDIIR LRDNG LLAKP THGDI IR AFYNN VLGEY EEYIT K
G2	NADP+-dependent isocitrate dehydrogenase	7.2/47000	6.53/46659	IIWEL IK NILGG TVFR SEGGF IWACK CATIT PDEKR TVEAE AAHGT VTR LVSGW VKPII IGR GQETS TNPIA SIFAW TR LIFPY VELDL HSYDL GIENR

	Fumarylacetoacetase	7.2/47000	6.92/46103	IGVAI GDQIL DLSVI K DIQQW EYVPL GPFLG K
	Phosphoglycerate kinase 1	7.2/47000	8.3/44596	ACANP AAGSV ILLEN LR ALESP ERPFL AILGG AK
G3	Isocitrate dehydrogenase	7.4/47000	6.53/46659	NILGG TVER LVSGW VKPII IGR LIFPV VELDL HSYDL GIENR
	Phosphoglycerate kinase 1	7.4/47000	8.3/44596	ACANP AAGSV ILLEN LR ALESP ERPFL AILGG AK
G4	Phosphoglycerate kinase 1	7.8/46000	8.3/44596	ELNYF AK SLLGK DVLFL K KDCVG PEVEK GCITI IGGGD TATCC AK ALESP ERPFL AILGG AK ACANP AAGSV ILLEN LR ITLPV DFVTA DKFDE NAK
G5	Phosphoglycerate kinase 1	8.6/45000	8.3/44596	ELNYF AK NNQIT NNQR YSLEP VAVEL K LGDVY VNDAF GTAHR VSHVS TGGGA SLELL EGK ALESP ERPEL AILGG AK ACANP AAGSV ILLEN LR ITLPV DFVTA DKFDE NAK
G6	47 kDa heat shock protein	9.5/53000	7.95/44527	GVVEV THDLQ K DTQSG SLLFI GR HLAGL GLTEA IDKNK LFYAD HPFIF LVR AVLSA EQLRD EEVHA GLGEL LR
G7	Similar to glucose regulated protein, 58 kDa	5.3/56000	5.32/36177	GYPTL LLFR
	Alpha-1-antitrypsin	5.3/56000	5.54/44792	SLLGN LGITK IAPNL ADFAF SLYR
G8	Similar to glucose regulated protein, 58 kDa	5.5/56000	5.32/36177	EEPGL AGVK GYPTL LWFR
	Alpha-1-antitrypsin	5.5/56000	5.54/44792	SLLGN ADFAF SLYR
G9	Alpha enolase	6.3/54000	6.99/47037	IEEEL GSK IGAEV YHNLK NVIK YNQIL RIEEE LGSK AAVPS GASTG IYEAL ELR SGETD DTFIA ELVVG LCTGQ IK DYPVV SIEDP FDQDD WEAWQ K DATNV GDEGG FAPNI LENKE ALELL K HIADL AGNAE VILPV PAFNV INGGS HAGNK SFIRD YPVVS IEDPF DQDDW EAWQK
	Beta enolase	6.3/54000	7.33/46855	GNPTV EVDLH TAK VNQIG SVTES IQACK AAVPS GASTG IYEAL ELR SGETE DTFIA DLVVG LCTGQ IK
	Phosphopyruvate hydratase	6.3/54000	4.63/16104	YISPD QLADL YK FTGSA GIQVV GDDLT VTNPK R AGYTD KVVIG MDVAA SEFYR DYPVV SIEDP FDQDD WEAWQ DATNV GDEGG FAPNI LENKE ALELL K SFIRD YPVVS IEDPF DQDDW EAWQK
G10	Alpha enolase	6.6/54000	6.99/47037	IEEEL GSK IGAEV YHNLK NVIK AAVPS GASTG IYEAL ELR SGETD DTFIA ELVVG LCTGQ IK DYPVV SIEDP FDQDD WEAWQ K DATNV GDEGG FAPNI LENKE ALELL K HIADL AGNAE VILPV PAFNV INGGS HANK SFIRD YPVVS IEDPF DQDDW EADDW EAWQK
	Beta enolase	6.6/54000	7.33/46855	GNPTV EVDLH TAK VNQIG SVTES IQACK

				AAVPS GASTG IYEAL ELR SGETE DTFIA DLVVG LCTGQ IK
	Phosphopyruvate hydratase	6.6/54000	4.63/16104	YISPD QLADL YK FTGSA GIQVV GDDLT VTNPK R AGYTD KVVIG MDVAA SEFYR DYPVV SIEDP FDQDD WEAWQ DATNV GDEGG FAPNI LENKE ALELL K SFIRD YPVVS IEDPF DQDDW EAWQK
G11	Alpha enolase	7/54000	6.99/47037	IEEEL GSK SKFGA NAILG VSLAV CK AAVPS GASTG IYEAL ELR YGKDA TNVGD EGGFA PNILE NK SGETD DTFIA ELVVG LCTGQ IK DYPVV SIEDP FDQDD WEAWQ K DATNV GDEGG FAPNI LENKE ALELL HIADL AGNAE VILPV PAFNV INGGS HANK SFIRD YPVVS IEDPF DQDDW EAWQK
	Beta enolase	7/54000	7.33/46855	GNPTV EVDLH TAK VNQIG SVTES IQACK SKFGA NAILG VSLAV CK AAVPS GASTG IYEAL ELR SGETE DTFIA DLVVG LCTGQ IK
	Phosphopyruvate hydratase	7/54000	4.63/16104	YISPD QLADL YK FTGSA GIQVV GDDLT VTNPK R AGYTD KVVIG MDVAA SEFYR YGKDA TNVGD EGGFA PNILE NK DYPVV SIEDP FDQDD WEAWQ K DATNV GDEGG FAPNI LENKE ALELL K SFIRD YPVVS IEDPF DQDDW EAWQK
G12	Ig heavy chain V region	7.8/55000	8.99/12975	LVESG GGLVQ PGGSL R
	Alpha enolase	7.8/55000	6.99/47037	AGYTD QVVIG MDVAA SEFYR SGETE DTFIA DLVVG LCTGQ IK
H1	Ig heavy chain V region	8.4/55000	8.99/12975	LVESG GGLVQ PGGSL R
	Protein disulphide isomerase A3	8.4/55000	5.78/54398	VVVAE NFDEI VNNEN K
H2	Ig heavy chain V region	8.1/55000	8.99/12975	LVESG GGLVQ PGGSL R
H3	Ig heavy chain V region	8.6/55000	8.99/12975	LVESG GGLVQ PGGSL R
H4	Ig heavy chain V region	8.8/55000	8.99/12975	LVESG GGLVQ PGGSL R
H5	Procollagen C-proteinase enhancer protein P	7/58000	7.55/45549	GFLLW YSGR ATSGT EHQFC GGR FCGTF RPAPL VAPGN QVTLR
	Beta-2-glycoprotein 1	7/58000	8.37/36254	VCPFA GILEN GAVR FICPL TGLWP INTLK
	Ig heavy chain V region	7/58000	8.99/12975	LVESG GGLVQ PGGSL R
H6	Ig heavy chain V region	7.4/56000	8.99/12975	LVESG GGLVQ PGGSL R
	Procollagen C-proteinase enhancer protein P	7.4/56000	7.55/45549	GFLLW YSGR
H7	Serum albumin	6/56000	5.86/66152	LSQRF PK TVLGN FAAFV QK RHPDY SVSLL LR DVFLG TFLYE YSR
	Procollagen C-proteinase enhancer protein P	6/56000	7.55/45549	YDALE VFAGS GTSGQ R FCGTF RPAPL VAPGN QVTLR
	Ig heavy chain V region	6/56000	8.99/12975	LVESG GGLVQ PGGSL R
	Aldehyde dehydrogenase, mitochondrial	6/56000	7.54/56708	YGLAA AVFTK ELGEY GLQAY TEVK
H8	Alpha-2-HS-glycoprotein	4.1/64000	5.4/36688	CNLLV EK HTLNQ VDSVK QDGQF SVLFA K CDSSP DSAED VHK QLTEH AVEGD CDFHV LK HSFSG VASVE SASGE AFHVG K GAQPS IPAAD GSVPV VRPCP GR HSFSG VASVE SASGE AFHVG KTPK EPACD DVETE QAALA AVDYI NK

				AQLVP LSASV SVEFA VAVTD CVAK
H9	Alpha-1-antichymotrypsin 3	4.4/62000	5.77/22840	LLPET LRR RIDAL YLPR VFTHE ADLSG VTGDN KLGVS
H10	Alpha-1-antichymotrypsin 3	4.5/64000	5.77/22840	RIDAL YLPR FSVSG DYQLK VFTHE ADLSG VTGDN KLGVS K
	Nucleobindin 1 (CALNUC)	4.5/64000	5.09/51087	DLELL IQTAT R
H11	Alpha-1-antichymotrypsin 3	4.6/62000	5.77/22840	LLPET LRR RIDAL YLPR FSVSG DYQLK DTLK VFTHE ADLSG VTGDN KLGVS K
	78 kDa glucose-regulated protein	4.6/62000	5.01/70478	ITPSY VAFTP EGER IINEP TAAAI AYGLD IEIES FYEGE DFSET LTR
	Fibromodulin	4.6/62000	5.56/41285	KVPDG LPSAL EQLYL EHNNV YSVPD SYFR
H12	Fibromodulin	4.7/64000	5.56/41285	SLILL DLSYN HLR IPPVN TNLEN LYLQG NR
	RAB GDP dissociation inhibitor alpha	4.7/64000	5/50565	TDDYL DQPCL ETINR SPYLY PLYGL GELPQ GFAR NPYYG GESSS ITPLE ELYKR
	78 kDa glucose-regulated protein	4.7/64000	5.01/70478	ITITN DQNR NGRVE IIAND QGNER TKPYI QVDIG GGQTK IINEP TAAAI AYGLD KR SQIFS TASDN QPTVT IK
	Alpha-1-antichymotrypsin 3	4.7/64000	5.77/22840	RIDAL YLPR VFTHE ADLSG VTGDN K
2A1	Serum albumin	6/65000	5.86/66152	LGEYG FQNAL IVR DVFLG TFLYE YSR KVPQV STPTL VEVAR LSCAE DYLSL VLNR SIHTL FGDKL CAIPS LR TCVAD ESAEN CDKSI HTLFG DK QNCEL FEKLG EYGFQ NALIV R LKPDP VALCA DFQED EQKFW GK CCAAP DHEAC FAVEG PKFVI EIR EFVEG TFTFH ADLCT LPEDE KQIK
2A2	Serum albumin	4.7/160000	5.86/66152	TVLGN FAAFV QK DVFLG TFLYE YSR RHPYF YAPEL LYYAI IYK
	Collagen alpha 1 (VI) chain	4.7/160000	5.23/106495	IALVI TDGR FLTAG RTDPA HDVR GLEQL LVGGS HLKEN K
2A3	Pyruvate kinase, M2 isozyme	8.6/63000	5.59/4863	GDLGI EIPAE K LDIDS PPITA R NTGII CTIGP ASR EAEAA IYHLQ LFEEL RR
	Serum albumin	8.6/63000	5.86/66152	RHPDY SVSLL LR DVFLG TFLYE YSR
2A4	Serum albumin	7.9/68000	5.86/66152	DVFLG TFLYE YSR
2A5	Vimentin	4.3/16000	6.47/30989	KLLEGE ESR FADLS EAANR ISLPL PNFSS LNLR QVQSL TCEVD ALKGT NESLE R
2A6	Cystatin B	5.7/15000	5.87/11130	VQVDD DDFVH LR VFESL PHENK PLTLS SYQTN K
	Haemoglobin beta chain	5.7/15000	7.25/16034	LLGNV IVVVL AR LLVVY PWTQR
2A7	Cartilage oligomeric matrix protein (COMP)	4/14000	4.35/80934	ELQET NAALQ DVREL LR
2A8	Haemoglobin beta chain	6.8/14000	7.25/16034	LLGNV IVVVL AR LLVVY PWTQR VNVDE VGGEA LGR

2A9	Polyubiquitin	7.3/14000	7.13/68497	EGIPP DQQR ESTLH LVLR TLSDY NIQK IQDKE GIPPD QQR TITLE VEPSD TIENV KAK TLSDY NIQKE STLHL VLR
2A10	Haemoglobin beta chain	6.7/14000	7.25/16034	VHLSA EEK EAVLG LWGK VLQSF SDGLK VVAGV ANALA HK LLVVY PWTQR VNVDE VGGEA LGR LGHDF NPNVQ AAFQK RLGHD FNPNV VLGLW GK LSELH CDQLH VDPEN FR
2A11	Haemoglobin beta chain	7.2/14000	7.25/16034	VLQSF SDGLK VVAGV ANALA LLGNV IVVVL AR LLVVY PWTQR VNVDE VGGEA LGR LGHDF NPNVQ AAFQK RLGHD FNPNV QAAFQ K VHLSA EEKEA VLGLW GK LSELH CDQLH VDPEN FR
2A12	Haemoglobin alpha chain	8.3/14000	8.76/15039	MFLGF PTTK VLSAA DKANV K FLANV STVLT SK VGGQA GAHGA EALER TYFPH FNLSH GSDQV K AVGHL DDLPG ALSAL SDLHA HK
	Haemoglobin beta chain	8.3/14000	7.25/16034	VLQSF SDGLK LLGNV IVVVL AR LLVVY PWTQR
2B1	Haemoglobin alpha chain	8.5/14000	8.76/15039	VLSAA DKANV K FLANV STVLT SK VGGQA GAHGA EALER
2B2	Haemoglobin alpha chain	9.5/14000	8.76/15039	MFLGF PTTK VLSAA DKANV K VGGQA GAHGA EALER TYFPH FNLSH GSDQV K AVGHL DDLPG ALSAL SDLHA HK
2B3	Haemoglobin beta chain	9.8/22000	7.25/16034	FLANV STVLT SK
2B4	Dihydropteridine reductase	7.9/32000	6.9/25803	EGGLL TLAGA K GAVHQ LCQSL AGK AALDG TPGMI GYGMA K
2B5	Collagen alpha 1 type II C-propeptide	7.3/32000	6.66/27405	KNWWS SK NSIAY LDEAA GNLKK ALLIQ GSNDV EIRAE GNSR
	Adenylate kinase isoenzyme 2, mitochondrial	7.3/32000	8.31/26365	NLETP PCK AVLLG PPGAG K NGFLL DGFPR GIHSA IDASQ TPDVV FASIL AAFSK
2B6	Glyceraldehyde 3-phosphate dehydrogenase	9.4/38000	8.52/35707	VKVGV NGFGR LEKPA KYDDI K GAAQN IIPAS TGAAK VPTPN VSVVD LTCR AITIF QERDP ANIK DGRGA AQNII PASTG AAK LISWY DNEFG YSNR IVSNA SCTTN CLAPL AK VIHDH FGIVE GLMTT VHAIT ATQK
	Ornithine decarboxylase	9.4/38000	5.28/51345	VTPFY AVKCN DSR
2B7	38 kDa heparin-binding glycoprotein (Gp38)	9.8/49000	9.17/42443	LLLSG AVSAG K GQGDA SSDRF SNADY AVSYV LR

2B8	Transaldolase	6.1/39000	6.36/37540	VSTEV DAR TIVMG ASFR ILDWH VANTD KK
	Serum albumin	6.1/39000	5.86/66152	QTALV ELLK TVLGN FAAFV QK
2B9	Actin, cytoplasmic 2	5.1/45000	5.31/41661	AGFAG DDAPR SYELP DGQVI TIGNE R VAPEE HPVLL TEAPL NPK
2B10	Gelsolin, plasma	4.9/53000	5.93/84775	TGAQE LLR EGGQT APAST R AGALN SNDAF VLK YIETD PANRD R AQPVQ VAEGS EPDSF WEALG GK TPSAA YLWVG TGASE AEKTG AQELL R
	Glutathione synthetase	4.9/53000	5.67/52384	ALAEG VLLR EGIAQ TVFLG LNR IEPEP FENCL LRPGS PAR
	Protein disulphide isomerase A6	4.9/53000	4.95/46170	LAAVD ATVNQ VLASR
2B11	Protein disulphide isomerase	4.3/59000	4.73/55223	ILEFF GLK THILL FLPK EADDI VNWLK LITLE EEMTK YQLDK DGVVL FK HNQLP LVIEF TEQTA PK LAKVD ATEES DLAQQ YGVR
2B12	Pigment epithelium-derived factor	5.7/53000	6.31/44056	LSYEG ELTK YGLDS DLNCK DTDTG ALLFI GK LAAAV SNFGY DLYR LTFPL DYHLN QPFIF VLR
	Transthyretin (prealbumin)	5.7/53000	6.34/13929	GSPAV NVGVK AADGT WEPFA LGK HYTIA ALLSP YSYST TALVS SPK
	Alpha enolase	5.7/53000	6.99/47037	IEEEL GSK AAVPS GASTG IYEAL ELR
2C1	RAB GDP dissociation inhibitor alpha	5.9/53000	5/50565	LYSES LAR GRDWN VDLIP K SPYLY PLYGL GELPQ GFAR FDLGQ DVIDF TGHAL ALYR
	Alpha enolase	5.9/53000	6.99/47037	VNQIG SVTES LQACK
2C2	Cytosol aminopeptidase	6.4/58000	6.29/52640	TLIEF LLR SAGAC TAAAF LK TKGLV LGIYS K GITFD SGGIS IK
	Leucine aminopeptidase	6.4/58000	7.61/56141	TLIEF LLR GITFD SGGIS IK
2C3	Matrix metalloproteinase-3 (MMP-3)	4.9/62000	5.59/52221	CGVPD VGYFS TFGL PK
	Alpha-1-antichymotrypsin 3	4.9/62000	5.77/22840	GLETP YFR FSVSG DYQLK DTLK
2C4	Vitamin D-binding protein	4.9/60000	5.22/51243	GQELC ADYSE NTFTE YK SCESN SPFPV HPGTA ECCTK
	Alpha-1-antitrypsin	4.9/60000	5.54/44792	IAPNL ADFAF SLYR
2C5	Antithrombin-III	5/62000	5.95/49039	RVWEL SK LQPLD FKENA EQSR LPGIV AEGRD DLYVS DAFHK
	60 kDa heat shock protein	5/62000	5.7/61054	AAVEE GIVLG GGCAL LR KPLVI IAEDV DGEAL STLVL NR
	Lysyl oxidase homolog 2	5/62000	5.92/84069	LGQGI GPIHL NEIQC TGNEK
2C6	Lysyl oxidase homolog 2	5.3/66000	5.92/84069	LGQGI GPIHL NEIQC TGNEK
2C7	Serum albumin	5.7/65000	5.86/66152	LSCAE DYLSL VLNR SIHTL FGDKL CAIPS LR EFVEG TFTFH ADLCT LPEDE K LKPDP VALCA DFQED EQKFW GK

				IYEAT LEDCC AKEDP PACYA TVFDK
	Prolyl 4-hydroxylase alpha subunit	5.7/65000	5.7/59109	SAWLS GYENP VVSR LNTEW SELEN LVLK ATISN PITGD LETVH YR
2C8	Collagen alpha 2 (XI) chain	7.5/105000	9.22/134012	TGPVG PAGLA GKPGP DGLR
2C9	Serotransferrin	6.7/95000	6.93/76967	YGYT GAFR
2C10	Serum albumin	6.1/95000	5.86/66152	QTALV ELLK TVLGN FAAFV QK *RHPDY SVSLL LR* KVPQV STPTL VEVAR VPQVS TPTLV EVARK
2C11	Serotransferrin	7/84000	6.93/76967	CDAKWS INSGG K TEGE CVNTP EK GSNFQ WNQLQ G DDKVT CVAEE LLK DLLFR DDTQC LAR IECVS AENTE DCIAK TTYES YLGAD YITAV ANLRK SAGWI IPMGL LYDQL PEPR RPVDD YENCY LAQVP SHAVV AR
	Inhibitor of carbonic anhydrase	7/84000	5.82/75697	RPVDD YENCY LAQVP SHAVV AR
	Lactotransferrin	7/84000	8.67/76143	YYGYT GAFR
2C12	Type IX collagen alpha 1 chain	9.7/14000	8.69/20906	VVGST ALQVA YK IGQDD LPGFD LISSQF QIDK ASR

Appendix 2

Identification of Proteins from Human Explant Medium by Mass Spectrometry

Proteins from the gel in fig 5.1 were identified by MALDI and ESI MS/MS

Spot	Protein	Exp pI/Mw	Theor. pI/Mw	Sequence
A1	Inhibin beta A	7.5/15000	7.07/12976	QFFVSFK
A2	Cytokine-like protein C17	4.5/18000	8.8/15577	LLQVSEPSEKEVR
A3	Cytokine-like protein C17	5.8/18000	8.8/15577	ALSQE ITR DFNLL QVSEP SEPCV ALSQE ITRDF NLLQV SEPSE PCVR
A4	Tetranectin	5/20000	5.8/19752	LDTLAQEVALLKEQQALQTVCLK CFLAFTQTKT
A5	Tetranectin	5.8/22000	5.8/19752	CFLAF TQTK LDTLA QEVAL LK TFHEA SEDCI SR GGTLS TPQTG SENDA LYEYL R
A6	Tetranectin	5.5/22000	5.8/19752	CFLAF TQTK EQQAL QTVCL K TENCA VLSGA ANKK TFHEA SEDCI SR SRLDT LAQEV ALLK GGTLS TPQTG SENDA LYEYL R LDTLA QEVALLKEQQ ALQTV CLK NWETE ITAQP DGGKT ENCAV LSGAA NGK
A7	Tetranectin	5/23000	5.8/19752	CFLAF TQTK EQQAL QTVCL K TENCA VLSGA ANGK TFHEA SEDCI SR SRLDT LAQEV ALLK IAYKN WETEI TAQPD GGK GGTLS TPQTG SENDA LYEYL R LDTLA QEVAL LKEQQ ALQTV CLK NWETE ITAQP DGGKT ENCAV LSGAA NGK
A8	Tetranectin	4.8/23000	5.8/19752	TFHEA SEDCI SR IAYKN WETEI TAQPD GGK GGTLS TPQTG SENDA LYEYL R
A9	Metalloproteinase inhibitor 2 (TIMP-2)	6.5/22000	6.5/21755	MHITLCDFIVPWDTLSTTQK
A10	Metalloproteinase inhibitor 2 (TIMP-2)	6.5/22000	6.5/21755	MHITLCDFIVPWDTLSTTQK
A11	Metalloproteinase inhibitor 1 (TIMP-1)	8.8/30000	8.5/20708	HLACLPR GFQALGDAADIR EPGLCTWQSLR FVYTPAMESVCGYFHR LQSGTHCLWTDQLLQGSEK TYTVGCEECTVFPCLSIPCK LQDGLLHITTCSFVAPWNSLSLAQR
A12	Metalloproteinase inhibitor 1 (TIMP-1)	8.5/30000	8.5/20708	HLACLPR GFQALGDAADIR EPGLCTWQSLR FVYTPAMESVCGYFHR LQSGTHCLWTDQLLQGSEK TYTVGCEECTVFPCLSIPCK LQDGLLHITTCSFVAPWNSLSLAQR
B1	Metalloproteinase inhibitor 1 (TIMP-1)	8/30000	8.5/20708	HLACLPR GFQALGDAADIR EPGLCTWQSLR FVYTPAMESVCGYFHR LQSGTHCLWTDQLLQGSEK TYTVGCEECTVFPCLSIPCK LQDGLLHITTCSFVAPWNSLSLAQR
B2	Metalloproteinase inhibitor 1 (TIMP-1)	7.5/30000	8.5/20708	HLACLPR GFQALGDAADIR EPGLCTWQSLR FVYTPAMESVCGYFHR LQSGTHCLWTDQLLQGSEK

				TYTVGCEECTVFPCLSIPCK
				LQDGLLHITTCSFVAPWNSLSLAQR
B3	Osteoinductive factor (OIF)	5/32000	5/26976	ESAYLYAR
				DFADIPNLR
				LPVLPPKLTLFNAK
				IEEIRLEGNPIVLGK
				LSLLEELSLAENQLLK
				LDFTGNLIEDIEDGTFSK
				RLDFTGNLIEDIEDGTFSK
				VIHLQFNNIASITDDTFCK
				LNNLTFLYLDHNALESVPLNLPESLR
B4	Clusterin	6/34000	5.9/50062	TLLSNLEEAK
				ASSIIDELFQDRFFT
				YVNKEIQNAVNGVK
B5	Clusterin	5.5/34000	5.9/50062	IDSLL ENDR
				TLLSN LEEAK
				ASSII DELFQ DRFFT R
B6	Osteoinductive factor	5.5/34000	5/26976	ESAYLYAR
				DFADIPNLR
				IEEIRLEGNPIVLGK
				RLDFTGNLIEDIEDGTFSK
				VIHLQFNNIASITDDTFCK
	Clusterin	5.5/34000	5.9/50062	ASSII DELFQ DRFFT
B7	Osteoinductive factor	5/36000	5/26976	ESAYLYAR
				DFADIPNLR
				IEEIRLEGNPIVLGK
				LSLLEELSLAENQLLKLPVLPPK
				RLDFTGNLIEDIEDGTFSK
				LNNLTFLYLDHNALESVPLNLPESLR
B8	Osteoinductive factor	5/36000	5/26976	LSLLEELSLAENQLLKLPVL
				DFADIPNLR
				LPVLPPKLTLFNAK
				IEEIRLEGNPIVLGK
				RLDFTGNLIEDIEDGTFSK
	Clusterin	5/36000	5.9/50062	ASSII DELFQ DRFFT
B9	Osteoinductive factor	5/36000	5/26976	ESAYLYAR
				DFADIPNLR
				LTAKDFADIPNLR
				LPVLPPKLTLFNAK
				IEEIRLEGNPIVLGK
				RLDFTGNLIEDIEDGTFSK
				VIHLQFNNINASITDDTFCK
				LSLLEELSLAENQLLKLPVLPPK
B10	Osteoinductive factor	5/36000	5/26976	ESAYL YAR
				DFADI PNLR
				LTAKD FADIP NLR
				LPVLP PKLTL FNAK
				IEEIR LEGNP IVLGK
				RLDFTG NLIED IEDGT FSK
				VIHLQ FNNIA SITDD TFCK
				LSLLE ELSLA ENQLL KLPVL PPK
	Clusterin	5/36000	5.9/50062	ASSII DELFQ DRFFT
B11	Osteoinductive factor	4.5/36000	5/26976	ESAYL YAR
				LTAKDFADI PNLR
				LPVLP PKLTL FNAK
				IEEIR LEGNP IVLGK
				RLDFT GNLIE DIEDG TFSK
				VIHLQ FNNIA SITDD TFCK
				LSLLE ELSLA ENQLL KLPVL PPK
B12	Osteoinductive factor	3.6/44000	5/26976	ESAYL YAR
				DFADI PNLR
				LTAKD FADIP NLR
				LPVLP PKLTL FNAK
	Osteonectin/SPARC	3.6/44000	4.7/30666	RLEAG DHPVE LLAR

				YIPPC LDSEL TEFPL R NVLVT LYERD EDNNL LTEK
C1	Osteonectin/SPARC	3.8/44000	4.7/30666	LEAGD HPVEL LAR TFDSS CHFFA TK YIPPC LDSEL TEFPL R FFETC DLDND KYIAL DEWAG CFGIK
C2	Vimentin	3.9/43000	5.1/46814	FLEQQ NK ILLAE LEQLK GQGK ISLPL PNFSS LNLR FADLS EAANR NNDAL R QVQSL TCEVD ALKGT NESLE R
	Osteonectin/SPARC	3.9/43000	4.7/30666	RLEAG DHPVE LLAR YIPPC LDSEL TEFPL R
C3	Vimentin	3.9/43000	5.1/46814	QQYES VAAK HLREY QDLLN VK KVESL QEETA FLK ILLAE LEQLK GQGK ISLPL PNFSS LNLR QVQSL TCEVD ALKGT NESLE R EEAEN TLQSF RQDVD NASLA R LLQDS VDFSL ADAIN TEFKN TR
C4	Vimentin	4/17000	5.1/46814	ISLPL PNFSS LNLR QVQSL TCEVD ALKGT NESLE R
C5	Vimentin	3.5/20000	5.1/46814	KVESL QEEIA FLK
C6	Vimentin	4/45000	5.1/46814	FLEQQ NK ILLAE LEQLK GQGK ISLPL PNFSS LNLR QVQSL TCEVD ALKGT NESLE R LLQDS VDFSL ADAIN TEFKN TR
C7	Osteoinductive factor	5.3/32000	5/26976	ESAYL YAR DFADI PNLR RLDFT GNLIE DIEDG TFSK
C8	Osteoinductive factor	4.8/34000	5/26976	ESAYL YAR LEGNP IVLGK DFADI PNLR LPVLP PKLTL FNAK LSLLE ELSLA ENQLL K LEGNP IVLGK HPNSF ICLK RLDFT GNLIE DIEG TFSK LNNLT FLYLD HNALE SVPLN LPESL R
C9	Clusterin	5/24000	5.9/50062	ELDESSLQVAER
C10	Clusterin	4.8/40000	5.9/50062	ASSII DELFQ DRFFT
	Cathepsin L	4.8/40000	5/31231	VFQEP LFYEA PR
C11	Lamin	5.8/42000	6.7/48452	IRIDS LSAQL SQLQK
C12	Clusterin	4.7/34000	5.9/50026	ELDES LQVAE R VTTVA SHTSD SDVPS GVTEV VVK
D1	Clusterin	4.9/34000	5.9/50062	ELDES LQVAE R ASSII DELFQ DR VTTVA SHTSD SDVPS GVTEV VVK
D2	Inhibin beta A	5.8/44000	7.9/45085	QPVPK FPVSS SIQR YVEIE DDIGR R AEMNE LMEQT SEIIT FAESG TAR
D3	Inhibin beta A	6/44000	7.9/45085	RPVDT QPVPK SELLL SEKVV DAR STWHV FPVSS SIQR VGENG YVEIE DDIGR
D4	Inhibin beta A	6.4/43000	7.9/45085	SELLL SEK RPDVT QPVPK LLDQG KSSLD VR STWHV FPVSS SIQR VGENG YVEIE DDIGR IACEQ CQESG ASLVL LGK AEMNE LMEQT SEIIT FAESG TARK

D5	Inhibin beta A	6.6/41000	7.9/45085	AEVWL FLKVP K SELLL SEKVV DAR STWHV FPVSS SIQR VGENG YVEIE DDIGR KSTWH VFPVS SSIQR IACEQ CQESG ASLVL LGKK
D6	Inhibin beta A	6.8/41000	7.9/45085	SELLL SEK RPDVT QPVPK LLDQG KSSLD VR STWHV FPVSS SIQR VGENG YVEIE DDIGR IACEQ CQESG ASLVL LGK
D7	Inhibin beta A	7/39000	7.9/45085	SELLL SEK STWHV FPVSS SIQR VGENG YVEIE DDIGR R IACEQ CQESG ASLVL LGK AEMNE LMEQT SEIIT FAESG TAR
D8	Inhibin beta A	7.3/39000	7.9/45085	SELLL SEK LLDQG KSSLD VR STWHV FPVSS SIQR VGENG YVEIE DDIGR R
D9	YKL-39/ Chitinase-3 like protein 2	7.5/39000	7.2/40871	LLLTA GVSAG R GNQWV GYDDV K LQDQQ VPYAV K SEVML YQTIN SLK SCNQG PYPLV QAVK LVCYF TNWSQ DR DKSEV MLYQT INSLK ENTHF TVLIH ELAEA FQK GPSSY YNVEY AVGYW IHK
D10	YKL-40/gp-39/Chitinase-3 like protein 1	8.2/41000	8.65/40476	QLLLS AALSA GK THGFD GLDLA WLYPG R EAGTL AYYEI CDFLR SFTLA SSETG VGAPI SGPGI PGRFT
D11	YKL-40/gp-39/ Chitinase-3 like protein 1	8.6/41000	8.65/40476	LVMGI PTFGR QLLLS AALSA GK THGFD GLDLA WLYPG R EAGTL AYYEI CDFLR THGFD GLDLA WLYPG RR GQEDA SPDRF SNTDY AVGYM LR SFTLA SSETG VGAPI SGPGI PGRFT K
	Fructose-bisphosphate aldolase	8.6/41000	8.4/36016	GILAA DESTG SIAK LQSIG TENTE ENRR IGEHT PSALA IMENA NVLAR GVVPL AGTNG ETTTQ GLDGL SER VDKGV VPLAG TNGETTTQGLDGLSER YASIC QQNGI VPIVE PEILP DGDH LKR
D12	Alpha-1-antitrypsin	4/63000	5.37/44324	FLENE DRR ITPNL AEFAF VTEEA PLK LSITG TYDLK SVLGQ LGITK
E1	YKL-40/Gp-39/ chitinase-3 like protein 1	9/40000	8.65/40476	VQYLK DR LVMGI PTFGR QLLLS AALSA GK EAGTL AYYEI CDFLR THGFD GLDLA WLYPG RR GQEDA SPDRF SNTDY AVGYM LR SFTLA SSETG VGAPI SGPGI PGRFT K
E2	YKL-40/Gp-39/ chitinase-3 like protein 1	9/40000	8.65/40476	THGFD GLDLA WLYPG RR
E3	Matrix metalloproteinase-3 (MMP-3)	5.2/58000	5.7/52221	ALKVW EEVTP LTFSR IVNYT PDLPK DAVDS AVEK
E4	Matrix metalloproteinase-3 (MMP-3)	4.8/59000	5.7/52221	FLGLE VTGK CGVPD VGHFR GIHTL GFPPT VR QIAED FPGID SK VWEEV TPLTF SR

				TYFFV EDKYW R ALKVW EEVTP LTFSR IVNYT PDLPK DAVDS AVEK
E5	Matrix metalloproteinase-3 (MMP-3)	4.4/59000	5.7/52221	SVLGQ LGITK LSITG TYDLK ITPNL AEFAF SLYR VFSNG ADLSG VTEEA PLK LSITG TYDLK SVLGQ LGITK
E6	Matrix metalloproteinase-2 (MMP-2)	4.5/70000	6.4/51844	TYIFA GDKFW R AFQVW SDVTP LR CGNPD VANYN FFPR GYPKP LTSLG LPPDV QR IIGYT PDLDP ETVDD AFAR TDKEL AVQYL NFFYG CPK
E7	ASPIC	4/84000	4.95/68434	TRFGA FAR SSPYY ALR LVNIA VDER GILAL RDVAA EAGVS K NNRWE DILSD EVNVA R
	Lumican	4/82000	6.17/36660	SLEYL DLSFN QIAR LDGNR ISETS LPPDM YECLR
E8	Lumican	4.5/53000	6.17/36660	LKEDA VSAAF K SLEDL QLTHN KITK SLEYL DLSFN QIAR LDGNR ISETS LPPDM YECLR
E9	Collagen alpha 1 type VI	4.5/130000	5.23/106495	VAVVQ YSGTG QQRPE R
E10	Complement factor B	5.8/90000	6.66/83000	ISVIR PSK VASYG VKPR GLTLR GSQR KDNEQ HVFK ALFVS EEEKK GDSGG PLIVH KR YGLVT YATYP K LEDSV TYHCS R YGQTI RPICL PCTEG TTR LLQEG QALEY VCPSG FYPYP VQTR
E11	Complement factor B	6.7/90000	6.66/83000	ISVIR PSK VASYG VKPR DISEV VTPR QKQVP AHAR KCLVN LIEK KDNEQ HVFK ALFVS EEEKK GDSGG PLIVH KR DNEQH VFKVK YGLVT YATYP K GTDYH KQPWQ AK QLNEI NYEDH KLK FLCTG GVSPY ADPNT CR VGSQY RLEDS VTYHC SR YGQTI RPICL PCTEG TTR LLQEG QALEY VCPSG FYPYP VQTR
E12	Complement factor B	6.2/90000	6.66/83000	LLQEG QALEY VCPSG FYPYP VQTR
F1	Complement factor B	6.7/88000	6.66/83000	LLQEG QALEY VCPSG FYPYP VQTR
F2	Complement factor B	6.9/61000	6.66/83000	YGLVT YATYP K
F3	Precursor polypeptide (AA-14 to 747)	9.3/150000	6.57/83881	FEKGP FGK SNEFD YPSVG QLAHK RSNEF DYPSV GQLAH RPGQA AAFNV TFR LALVG LLSLG CVLSQ ECTK DSEGC WVAYT LEQQD GMDR QLSPQ KVTLY LRPGQ AAAFN VTFR GDCDG VQINV PITFQ VKVTA TECIQ EQSFVIR
	Kinesin-like protein	9.3/150000	5.42/138630	MASQP NSSAK

				HSDKL NGNLE K LDIPT GTTPQ RK KSYLY PSTLV R SHSVF SVTIH MK SKVEE TTEHL VTK QCGNL TEDLK TIK CESLN TRTVY FSEQW VSSLN ER TRTSI IATIS PASLN LEETL STLEY AHR
F4	Putative DNA/chromatin binding motif	5.5/170000	5.90/144676	LPEGD ALR SEKPP LEK EFGSG FPVR DMAIM IEDEK SQNQL TVNEL R ADVLD VVVAS TVQK TNQCA GEFVI TFPR EIEEI PAYLP NGAAL KDSVQ R
F5	Cartilage intermediate layer protein (CILP)	7.5/60000	8.73/132538	VIPQG SCRR IVGPL EVNVR CFDGT SDGSS R ACEEA PPSAA HFR SNVGV ALTFN CVER FNPNA IGVPQ PYLNK DRDQP NVSAA CLEFK FNPNA IGVPQ PYLNK LNYR QSAFQ YLQST PAQSP AAGTC QGR SERFL PSEQI QGVVI SVINL EPR
F6	Cartilage intermediate layer protein (CILP)	7.8/60000	8.73/132538	TGFLS NPR VIPQG SCR IVGPL EVNVR TFLVG NLEIR ACEEA PPSAA HFR SNVGV ALTFN CVER FNPNA IGVPQ PYLNK LNYR FLPSE QIQGV VISVI NLEPR QSAFQ YLQST PAQSP AAGTV QGR
F7	Cartilage intermediate layer protein (CILP)	8.2/59000	8.73/132538	TGFLS NPR VIPQG SCR IVGPL EVNVR TFLVG NLEIR ACEEA PPSAA HFR SNVGV ALTFN CVER DRDQP NVSAA CLEFK FNPNA QIQGV VISVI NLERP FLPSE QIQGV VISVI NLEPR QSAFQ YLQST PAQSP AAGTV QGR
F8	Cartilage intermediate layer protein (CILP)	8.7/59000	8.73/132538	IVGPL EVNVR TFLVG NLEIR CFDGT SDGSS R RLFNL DVPES RR ACEEA PPSAA HFR FNPNA IGVPQ PYLNK DRDQP NVSAA CLEFK FLPSE QIQGV VISVI NLEPR QSAFQ YLQST PAQSP AAGTV QGR
F9	Cartilage intermediate layer protein (CILP)	8.8/61000	8.73/132538	VIPQG SCR IVGPL EVNVR TFLVG NLEIR CFDGT SDGSS ACEEA PPSAA HFR SNVGV ALTTFN CVER FNPNA IGVPQ PYLNK DRDQP NVSAA CLEFK FLPSE QIQGV VISVI NLEPR QSAFQ YLQST PAQSP AAGTV QGR
F10	Cartilage intermediate layer protein (CILP)	9/62000	8.73/132538	TGFLS NPR

				VIPQG SCR IVGPL EVNVR TFLVG NLEIR ACEEA PPSAA HFR FNPNA IGVPQ PYLNK LNYR FLPSE QIQGV VISVI NLEPR QSAFQ YLQST PAQSP AAGTV QGR
F11	Cartilage intermediate layer protein (CILP)	9.2/62000	8.73/132538	TGFLS NPR VIPQG SCR TAKEI ALGR VIPQG SCRR IVGPL EVNVR QSGVV ASLRF PR SNVGV ALTFN CVER FNPNA IGVPQ PYLNK QSAFQ YLQST PAQSP AAGTV QGR
F12	Golgin-160	8.6/50000	5.74/66349	LDSEL KELR EAADA ELGQL R HLVQA LQASL EK QELMQ VHGEK R GKLTG LGQSN AALR VIELE DELQE SR ELEGL QQLLQ NVK ERDEM ETHLQ SLQFD K EHLQV KLQAE ADDLQ IR
G1	Golgin-160	9.5/54000	5.74/66349	LGSDL TSAQK EAADA ELGQL R QELMQ VHGEK R LGSDL TSAQK EMK VLELE DELQE SR ELEGL QQLLQ NVK ERDEM ETHLQ SLQFD K ELQAK EHLVQ KLQAE ADDLQ
G2	L-lactate dehydrogenase	8.6/34000	8.46/36557	SADTL WGIQK VTLTS EEEAR VIGSG CNLDS AR NVNIF KFIIP NVVK
G3	L-lactate dehydrogenase	8.2/34000	8.46/36557	VIGSG CNLDS AR
G4	Collagen alpha 1 type II C-propeptide	7/33000	6.66/27405	QHDAE VDATL K FTYTA LKDGC TK NSIAY LDEAA GNLKK
G5	Collagen alpha 1 type II C-propeptide	6.6/33000	6.66/27405	SLNNQ IESIR ALLIQ GSNDV EIR
G6	Glyceraldehyde 3-phosphate dehydrogenase	8.2/36000	8.58/35922	GALQN IIPAS TGAAK
G7	Glyceraldehyde 3-phosphate dehydrogenase	8.4/36000	8.58/35922	GALQN IIPAS TGAAK
G8	Glyceraldehyde 3-phosphate dehydrogenase	8.7/36000	8.58/35922	GALQN IIPAS TGAAK VPTAN VSVVD LTCR IISNA SCTTN CLAPL AK
G9	Glyceraldehyde 3-phosphate dehydrogenase	8.8/36000	8.58/35922	GALQN IIPAS TGAAK VPTAN VSVVD LTCR IISNA SCTTN CLAPL AK VIHDN FGIVE GLMTT VHAIT ATQK
G10	Glyceraldehyde 3-phosphate dehydrogenase	9.2/36000	8.58/35922	GALQN IIPAS TGAAK LVING NPITI FQERD PSK
G11	Procollagen C-proteinase enhancer protein P	6.2/50000	7.55/45549	TGGLD LPSPP TGASL K ATSGT EHQFC GGRLE K FCGTF RPAPL VAPGN QVTLR
G12	Procollagen C-proteinase enhancer protein P	6.5/47000	7.55/45549	GFLLW YSGR VFDLE LHPAC ATSGT EHQFC GGR TGGLD LPSPP TGASL K YDALE VFAGS GTSGQ R YDSVS VFNGA VSDDS RR FCGTF RPAPL VAPGN QVTLR

				VFDLE LHPAC RYDAL EVFAG SGTSG QR
H1	Procollagen C-proteinase enhancer protein P	6.8/47000	7.55/45549	GFLLW YSGR ATSGT EHQFC GGR TGGLD LPSPP TGASL K TDALE VFAGS GTSGQ R ATSGT EHQFC GGRLE K YDSVS VFNGA VSDDS RR FCGTF RPAPL VAPGN QVTLR VFDLE LHPAC RYDAL EVFAG SGTSG QR
H2	Procollagen C-proteinase enhancer protein P	7.3/47000	7.55/45549	GFLLW YSGR TGGLD LPSPP TGASL K YDALE VFAGS GTSGQ R EPGEG LAVTV SLIGA YK YDSVS VFNGA VSDDS R GESGY VASEG FPNLY PPNK FCGTF RPAPL VAPGN QVTLR
H3	Procollagen C-proteinase enhancer protein P	7.5/47000	7.55/45549	TGGLD LPSPP TGASL K ATSGT EHQFC GGRLE K FCGTF RPAPL VAPGN QVTLR
H4	Procollagen C-proteinase enhancer protein P	9.5/19000	7.55/45549	TGGLD LPSPP TGASL K ATSGT EHQFC GGRLE K FCGTF RPAPL VAPGN QVTLR
H5	Extracellular superoxide dismutase [Cu-Zn]	5.7/31000	6.32/24162	GGNQA SVENG NAGR AVVVH AGEDD LGR AGLAA SLAGP HSIVG R LACCV VGVCG PGLWE R LACCV VGVCG PGLWE R
H6	Extracellular superoxide dismutase [Cu-Zn]	6/31000	6.32/24162	VTGVV LFR GGNQA SVENG NAGR AGLAA SLAGP HSIVG R LACCV VGVCG PGLWE R
H7	Extracellular superoxide dismutase [Cu-Zn]	6.5/30000	6.32/24162	VTGVV LFR AVVVH AGEDD LGR AGLAA SLAGP HSIVG R LACCV VGVCG PGLWE R RLACC VVGVC GPGLW ER
H8	Carbonic anhydrase	6.8/29000	6.63/28739	VLDAL QAIK ESISV SSEQL AQFR LYPIA NGNNQ SPVDI K
H9	Carbonic anhydrase	7/29000	6.63/28739	VLDAL QAIK GGPFS DSYR YSSLA EAASK ADGLA VIGVL MK ESISV SSEQL AQFR LYPIA NGNNQ SPVDI
H10	Complement factor D	7.2/24000	6.85/24404	RPDSL QHVLL PVLDR DSCKG DSGGP LVCGG VLEGV VTSGS R
H11	Complement factor D	7.2/23000	6.85/24404	RPDSL QHVLL PVLDR VDRDV APGTL CDVAG WGIN HAGR DSCKG DSGGP LVCGG VLEGV VTSGS AVPHP DSQPD TIDHD LLLLQ LSEK
H12	Complement factor D	7.5/24000	6.85/24404	RTHHD GAITE R ATLGP AVRPL PWQR RPDSL QHVLL PVLDR DSCKG DSGGP LVCGG VLEGV VTSGS R AVPHP DSQPD TIDHD LLLLQ LSEK
2A1	Biliverdin reductase B	7.7/23000	7.13/22119	VLRES GLK LQAVT DDHIR NDLSP TTVMS EGAR TVAGQ DAVIV LLGTR VISKH DLGHF MLR CLTTD EYDGH STYPS HQYQ
2A2	Phosphatidylethanolamine-binding protein	7.8/22000	7.43/20925	VLTPT QVK NRPTS ISWDG LDSGK

				YVWLV YEQDR PLKCD EPILS NR
2A3	Serum amyloid P	5.2/27000	6.12/23258	DNELL VYKER QGYFV EAQPK VGEYS LYIGR HK GLRQG YFVEA QPK HTDLS GKVFV FPR IVLGQ EQDSY GGKFD R AYSLF SYNTQ GRDNE LLVYK
2A4	Cartilage oligomeric matrix protein (COMP)	4.3/31000	4.35/80934	AVAEP GIQLK SSTGP GEQLR NALWH TGDTE SQVR WFLQH RPQVG YIR FYEGP ELVAD SNVVL DTTMR GGR AFQTV VLDPE GDAQI DPNWV VLNQG
2A5	Cartilage oligomeric matrix protein (COMP)	9.6/21000	4.35/80934	SSTGP GEQLR NALWH TGDTE SQVR
2A6	α-1-microglobulin-bikunin	4.5/32000	6.32/35528	ETLLQ DFR WYNLA IGSTC PWLK IYGKW YNLAT GSTCP WLK
	Cartilage intermediate layer protein (CILP)	4.5/32000	8.73/132538	FLPSE QIQGV VISVI NLEPR
	Cartilage oligomeric matrix protein (COMP)	4.5/32000	4.35/80934	AVAEP GIQLK AFQTV VLDPE GDAQI DPNWV VLNQG R
2A7	Cartilage oligomeric matrix protein (COMP)	5/30000	4.35/80934	LLWKD PR SSTGP GEQLR
2A8	Peroxiredoxin 2	5.3/225000	5.66/21891	TDEGI AYR EYFSK HN IGKPA PDFK RLSEDY GVLK QITVN DLPVG GLFIII DGKGV LR ATAVV DGAFK EVK GVLRQ ITVND LPVGR KEGGLG PLNIP LLADV TRR LSEDY GVLKT DEGIA YR
2A9	Peroxiredoxin 2	7.6/22000	5.66/21891	IGKPA PDFK ATAVV DGAFK EVK GVLRQ ITVND LPVGR
2A10	Destrin (Actin-depolymerizing factor)	8/18000	8.06/18505	YALYD ASFET K
2A11	Transthyretin (prealbumin)	5.2/16000	5.35/13761	AADDT WEPFA SGK ALGIS PFHEH AEVVF TANDS GPR RYTIA ALLSP YSYST TAVVT NPK TSESG ELHGL TTEEQ FVEGI YKVEI DTK
2A12	Peptidyl-prolyl cis-trans isomerase B	9.7/20000	9.25/20289	TVNF VALAT GEKD DTNGS QFFIT TVK
2B1	Peptidyl-prolyl cis-trans isomerase	8/17000	7.82/17881	FEDEN FILK VSFEL FADKV PK SIYGE KFEDE NFILK VNPTV FFDIA VDGEP LGR
2B2	Chain A, zinc dependent dimers	9.2/24000	9.1/19561	LQDLY SIVRR IFSFD GKDVL R GADFQ CFQQA R SVWHG SDPNG RP RLTES YCETW R AVGLA GTFRA FLSSR GIRGA DFQCF QQAR AFLSS RLQDL YSIVR LTESY CETWR TEAPS ATGQA SSLLG GR
	Type XVIII collagen	9.2/24000	9.3/17732	GYPGIPGPK AVGLAGTFR LQDLYSIVRR SVWHGSDPNGR IFSFDGKDVLR GADFQCFQQAR LTESYCETWRS

				SVWHGSDPNGRR DVLRPTWPQK RLTESYCETWR (data limited)
2B3	Haemoglobin alpha chain	9.3/14000	8.73/15136	MFLSF PTTK VLSPA DKTNV K FLASV STVLT SK TYFPH FDLSH GSAQV K
2B4	Haemoglobin alpha chain	8.7/14000	8.73/15136	FLASV STVLT SK TYFPH FDLSH GSAQV K
2B5	Haemoglobin alpha chain	8.5/14000	8.73/15136	VHLTP EEK TAVNA LWGK VVAGV ANALA HK VNVDA VGGEA LLVVY PWTQR LLGVN LVCVL AR VLGAF SDGLA HLDNL K
2B6	Lysozyme C	9.5/14000	9.38/16537	VVRDP QGIR STDYG IFQIN SR ATNYN AGDRS TDYGI FQINS
2B7	Profilin 1	8.8/14000	8.47/14923	STGGA PTFNV TVTK DSLLQ DGEFS MDLR TFVNI TPAEV GVLVG KDR
2B8	Haemoglobin delta chain	8/14000	7.97/15924	VHLTP EEK LHVDP ENFR VVAGV ANALA HK VNVDA VGGEA LGR LLVVY PWTQR LLGNV LVCVL AR KVLGA FSDGL AHLDN LK
2B9	Haemoglobin beta chain	7.5/14000	6.81/15867	SAVTA LWGK VHLTP EEK LHVDP ENFR VVAGV ANALA HK LLVVY PWTQR VNVDE VGGEA LGR EFTPP VQAAY QK GTFAT LSELH CDK VLGAF SDGLA HLDNL K LLGNV LVCVL AHHFG K KVLGA FSDGL AHLDN LK
2B10	Haemoglobin beta chain	7.2/14000	6.81/15867	SAVTA LWGK VHLTP EEK LHVDP ENFR VVAGV ANALA HK LLVVY PWTQR VNVDE VGGEA LGR EFTPP VQAAY QK LLGNV LVCVL AHHFG K KVLGA FSDGL AHLDN LK GTFAT LSELH CDKLH VDPEN FR
2B11	Haemoglobin beta chain	6.7/14000	6.81/15867	SAVTA LWGK VHLTP EEK LHVDP ENFR VVAGV ANALA HK LLVVY PWTQR VNVDE VGGEA LGR VLGAF SDGLA HLDNL K
2B12	Haemoglobin delta chain	6/14000	7.97/15924	LLVVY PWTQR???
2C1	Haptoglobin-1	5.5/20000	5.23/9192	TEGDG VYTLN NEKQW INK
2C2	Retinoic acid receptor responder protein 2	9/18000	8.99/16856	LGSED KVLGR GLQVA LEEFH K LVHCP IETQV LR EAEEH QETQC LR AGEDP HSFYF PGQFA FSK LVHCP IETQV LREAE EHQET QCLR

2C3	Retinoic acid receptor responder protein 2	9.3/18000	8.99/16856	KCLAC IK LVHCP IETQV LR EAEEH QETQC LR AGEDP HSFYF PGQFA FSK
2C4	Thrombospondin 4 N-terminal domain	9.8/21000	9.23/29230	KPQDF LEELK AFAGP SQKPE TIELR KPQDF LEELK LVVR GAGSL ELYLD CIQVD SVHNL PR
2C5	Ribonuclease pancreatic precursor	8.8/21000	8.98/14574	YPNCA YR CKPVN TFVHE PLVDV QNVCF QEK
2C6	Glutathione S-transferase	5.2/23000	5.44/23224	ASCLY GQLPK PPYTV VYFPV R FQDGD LTLYQ SNTIL R ALPGQ LKPEE TLLSQ NQGGK
2C7	Prolargin	5.2/29000	9.45/41646	KVPVI PPR NQLEE VPSAL PR VLEKL PGLVF LYMEK ECYCP PDFPS ALYCD SR
2C8	Prolargin	5.7/29000	9.45/41646	KVPVI PPR NQLEE VPSAL PR VLEKL PGLVF LYMEK ECYCP PDFPS ALYCD SR
2C9	Phosphoglycerate mutase	6.3/26000	6.67/28803	HYGGL TGLNK ALPFW NEEIV PQIK SYDVP PPPME PDHPF YSNIS K YADLT EDQLP SCESL KDTIA R
2C10	Ig kappa chain region	8.5/24000	5.58/11608	SGTAS VVCLL NNFYP R VYACE VTHQG LSSPV TK TVAAP SVFIF PPSDE QLK
2C11	Ig kappa chain region	9/24000	5.58/11608	SGTAS VVCLL NNFYP R TVAAP SVFIF PPSDE QLK
2C12	Chondroadherin	9.4/29000	9.43/38271	ASRPD ATCAS PAC SIPDN AFQSF GR FSDGA FLGVT TLK VVEEL KLSHN PLK YLETL WLDNT NLEK GLLSP LVNLF ILQLN NNK FHVDR NQLSS YPSAA LSK LNQLP SNFPF DSLET LALTN NPWK AGAFD DLTEL TYLYL DHNKV TELPR
2D1	Chondroadherin	9.5/33000	9.43/38271	LRVVE ELK ASRPD ATCAS PAK SIPDN AFQSF GR NQLSS YPSAA LSK QLIYL YLSHN DIR YLETL WLDNT NLEK GLLSP LVNLT ILQLN NNK LNQLP SNFPF DSLET LALTN NPWK AGAFD DLTEL TYLYL DHNKV TELPR
2D2	Annexin II	8.6/33000	7.65/38472	TNQEL QEINR GVDEV TIVNI LTNR SALSG HLETV ILGLL K GLGTD EDSLI EIICS R
2D3	Annexin II	7.7/34000	7.65/38472	DLYDA GVKR QDIAF AYQR TNQEL QEINR SLYYY IQQDT K GVDEV TIVNI LTNR TKGVD EVTIV NILTN R RAEDG SVIDY ELIDQ DAR AYTNF DAERD ALNIE TAIK
2D4	Complement C4	6.8/32000	6.37/330373	LLATL CSAEV CQCAE GKCPR
2D5	Hypothetical 187.1 kDa protein in OGG1-CNA2	4.4/14000	8.16/187132	SFVNI VAK
2D6	Ribonuclease 4	9.3/17000	9.18/13823	SICST TNIQC K

				QHVHP EETGG SDR FNTFI HEDIW NIR
2D7	Cofilin	8.4/19000	8.22/18502	YALYD ATYET K KEDLV FIFWA PESAP LK
2D8	C-type lectin	7.5/18000	9.15/19840	CYKLAS EGLK EIQAL QTVCL R *NSDEI* NALQD YGKR LWTEV NALKE IQALQ TVCLR
2D9	Superoxide dismutase [Cu-Zn]	5.5/19000	6.83/18995	ESNGP VKVWG SIK ADDLG KGGNE ESTK GDGPV QGIIN FEQK AVCVL KGDGP VQGII NFEQK
2D10	Apolipoprotein A-I	4.8/23000	5.27/28078	AELQE GAR AKPAL EDLR ATEHL STLSE K QGLLP VLEF K VQPYL DDFQK THLAP YSDEL R VSFLS ALEEY TK DYVSQ FEGSA LGK VKDLA TVYVD VLK LLDNW DSVTS TFSK
2D11	Apolipoprotein A-I	4.5/23000	5.27/28078	AELQE GAR QGLLP VLESF K VQPYL DDFQK VSFLS ALEEY TK LLDNW DSVTS TFSK
2D12	Triosephosphate isomerase	6.7/24000	7.09/26581	IAVAA QNCYK SNVSD AVAQS TR TATPQ QAQEV HEK
2E1	Triosephosphate isomerase	7/24000	7.09/26581	FFVGG NWK IAVAA QNCYK SNVSD AVAQS TR TATPQ QAQEV HEK DCGAT WVVLG HSER VVLAY EPVWA IGTGK VPADT EVVCA PPTAY IDFAR
2E2	Complement C1q subcomponent, B chain	9.1/30000	8.85/23741	*TINVP LRR* LEQGE NVFLQ ATDK
2E3	Collagen alpha 1 (XI) chain	7.2/29000	9.1/97667	TTGFC TNR AIVDT NGITV FGTR
2E4	Annexin V	4.3/33000	4.94/35805	QEISA AFK SEIDL FNIR ETSGN LEQLL LAVVK GLGTD EESIL TLLTS R
2E5	Annexin V	8.3/32000	4.94/35805	GLGTD EDTLI EILAS R GGPGS AVSPY PTFNP SSDVA ALHK
2E6	Fibrogen beta chain	5.3/40000	7.95/50762	EDGGG WWYNR NYCGL PGEYW LGNDK TPCTV SCNIP VVSGK ECEEI IR
2E7	Pigment epithelium-derived factor	6.7/42000	6.56/40071	LAAAV SNFGY DLYR KTSLE DFYLD EER
2E8	Pigment epithelium-derived factor	5.1/50000	6.56/40071	YGLDS DLSCK ELLDT VTAPQ K DTDTG ALLFI GK LQSLF DSPDF SK LAAAV SNFGY DLYR KTSLE DFYLD EER LDLQE INNWV QAQMK EIPDE ISILL LGVAH FK ALYYD LISSP DIHGT YKELL DTVTA PQK
2E9	Pigment epithelium-derived factor	5.3/49000	6.56/40071	DTDTG ALLFI GK LQSLF DSPDF SK LAAAV SNFGY DLYR

				KTSLE DFYLD EER EIPDE ISILL LGVAH FK ALYYD LISSP DIHGT YKELL DTVTA PQK
2E10	Pigment epithelium-derived factor	5.6/48000	6.56/40071	SSFVA PLEK YGLDS DLSCK IKSSF VAPLE K DTDTG ALLFI GK LQSLF DSPDF SK LAAAV SNFGY DLYR KTSLE DFYLD EER ALYYD LISSP DIHGT YKELL DTVTA PQK
2E11	Pigment epithelium-derived factor	5.7/47000	6.56/40071	SSFVA PLEK TVQAV LTVPK YGLDS DLSCK IKSSF VAPLE K LKLSY EGEVT K LQSLF DSPDF SK LAAAV SNFGY DLYR KTSLE DFYLD EER ALYYD LISSP DIHGT DTVTA PAK
2E12	Pigment epithelium-derived factor	5.8/46000	6.56/40071	YGLDS DLSCK DTDTG ALLFI GK LQSLF DSPDF SK LAAAV SNFGY DLYR KTSLE DFYLD EER
2F1	Phosphoglycerate kinase	8.7/43000	8.3/44596	FCLDN GAK NNQIT NNQR SLLGK DVLFL K YSLEP VAVEL K LGDVY VNDAF GTAHR VSHVS TGGGA SLELL EGK DVLFL KDCVG PEVEK ALESP ERPEL AILGG AK ACANP AAGSV ILLEN LR ITLPV DFVTA DKFDE NAK
2F2	Serine protease HTRA1	7.3/55000	7.89/49048	LPVLL LGR LHRPP VIVLQ R IAPAV VHIEL FR CPPQP EHCEG GR GACGQ GQEDP NSLR VTAGI SFAIP SDKIK SAPLA AGCPD RCEPA R
2F3	Ig gamma-2 chain C region	7.6/55000	7.66/35884	NQVSL TCLVK VVSVL TVVHQ DWLNG KEYK GFYPS DIAVE WESNG QPENN YK
2F4	Complement C3 β-chain	7.2/68000	8.7/70420	SGIPI VTSPY QIHFT K
2F5	Serotransferrin	6.9/71000	6.7/75181	CLVEK GDVAF VK SVIPES DGPSV ACVK MYLGY EYVTA IR SAGWN IPIGL LYCDL PEPR
2F6	Serotransferrin	6.7/72000	6.7/75181	EGYYG YTGAF R SASDL TWDNL K CSTSS LLEAC TFR TAGWN IPMGL LYNK KPVEE YANCH LAR EDPQT FYYAV AVVK EGTCP EAPTD ECKPV K NLNEK DYELL CLDGT R EDLIW ELLNQ AQEHF GK SAGWN IPIGL LYCDL PEPR
2F7	Serotransferrin	6.4/73000	6.7/75181	APNHA VVTR KASYLDCIR SADL TWDNL K SVIPS DGPSV ACVK MYLGY EYVTA IR

				CSTSS LLEAC TFR DLLFR DDTVC LAK SAGWN IPIGL LYCDL PEPR
2F8	Serotransferrin	6.5/74000	6.7/75181	APNHA VVTR KASYL DCIR SVIPS DGPSV ACVK KPVEE YANCH LAR
2F9	Fibromodulin	3.6/50000	5.57/40988	YLPFV PSR SLILL DLSYN HLRK
2F10	Fibromodulin	3.5/60000	5.57/40988	YLPFV PSR SLILL DLSYN HLR SLREL HLDHN QISR IPPVN TNLEN LYLQG NR
2F11	Alpha-1B glycoprotein	4.6/75000	5.65/51940	LLELT GPK LETPD FQLFK SGLST GWTQL SKLLE LTGPK CEGPI PDVTF ELLRE GETK TPGAA ANLEL IFVGP QHAGN YR
2F12	Hemopexin	5.2/73000	6.43/49295	RLWWL DLK YYCFQ GNQFL R EVGTP HGILL DSVDA AFICP GSSR
2G1	Annexin I	6.5/35000	6.64/38583	GLGTD EDTLI EILAS R
2G2	Related to glycerol-3-phosphate dehydrogenase	6.7/38000	7.6/76907	VRLIG AHGFS R
2G3	H factor (complement)-like 1	6.8/39000	7.39/37650	STDTS CVNPP TVQNA HILSR
2G4	Actin, aortic smooth muscle (α-actin 2)	4.7/43000	5.24/41774	SYELP DGQVI TIGNE R
2G5	Procollagen C-endopeptidase enhancer	7.6/52000	7.41/47972	FCGTF RPAPL VAPGN QVTLR
2G6	Hypothetical 72.2 kDa protein	8/51000	04/72247	VFLVG NVEIR ECQGA PVTAS HFR VEADK YEYNV VPFR SLREC QGAPV TASHF R
2G7	Hypothetical 72.2 kDa protein	9.4/54000	04/72247	LAKEI AIGR LFNLD VPER VFLVG NVEIR VEADK YEYNV VPER SLREC QGAPV TASHF R AYAND KFTPS EQVEGVVVTLVNLEPAPGFSANPR
2G8	Hypothetical 72.2 kDa protein	8.9/53000	8.04/72247	LAKEI AIGR VFLVG NVEIR RLFNL DVPER VEADK YEYNV VPFR SLREC QGAPV TASHF R
2G9	Ig gamma-4 chain C region	8/54000	7.81/35940	GPSVT PLAPC SR
2G10	Beta-2-glycoprotein I	6.6/57000	8.37/36254	KATVV YQGER VCPFA GILEN GAVR FICPL TGLWP INTLK ATFGC HDGYS LDGPE EIECT K CPFPS RPDNG FVNYP AKPTL YYK YTTFE YPNTI SFSCN TGFYL NGADS AK
2G11	Ig gamma-1 chain C region	8.2/90000	8.46/36105	GPSVF PLAPS SK
2G12	Polynucleotide nucleotidyltransferase	5.3/22000	5.3/79170	QAAGA VLMRQ GK