The MMP-2/MT1-MMP/TIMP-2 Enzyme System In Abdominal Aortic Aneurysm Disease

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Declaration

I hereby declare that the work contained in this thesis is entirely my own, except where acknowledged, and has not been submitted for a degree to this or any other University.

Signature: M. Acutter

1999

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Summary

Abdominal aortic aneurysm (AAA) represents a complex biochemical pathology, characterised by marked tissue remodelling and severe inflammation. The weakening of the arterial wall is associated with elevated production of matrix metalloproteinases (MMPs), enzymes that degrade extracellular matrix components causing a reduction in tensile strength and mechanical integrity. However, the cause of this overproduction of MMPs remains poorly understood.

Analysis of small AAAs has demonstrated that MMP-2 is the most prominent MMP, an enzyme known to be produced by most mesenchymal cells. The present thesis investigated the hypothesis that aortic smooth muscle cells (SMCs) are the source of excess MMP-2, which may degrade elastin fibres and initiate the degenerative and inflammatory process characteristic of aneurysmal disease.

Histological analysis of control and aneurysm tissue was followed by focused testing of the hypothesis, by isolating and comparing SMCs from control and aneurysmal aortas. Aneurysmal SMCs expressed significantly more MMP-2 than control cells, but levels of MT1-MMP and TIMP-2 were not significantly different. MMP-2 was shown to be capable of inducing elastolytic changes characteristic of aneurysm disease in a porcine *in vitro* model of AAA.

Similar methods suggested that this was not the case in dermal fibroblasts, providing evidence that the elevated level of MMP-2 in SMCs was not reflected in all mesenchyme-derived tissues. Published evidence suggested that AAA may be a local manifestation of a generalised dilating process, and this hypothesis was addressed by examining the MMP expression of vascular tissue remote from the aneurysm site. Inferior mesenteric vein from AAA patients produced elevated levels of MMP-2 compared to control vein, suggesting that this phenomenon may be systemic but vascular tissue-specific in nature. These data suggest that AAA patients may be predisposed to vascular elastolysis, manifesting itself in later life as aneurysmal dilatation of the aorta. In conclusion, a novel model of AAA aetiology is proposed.

Contents

List Of Abbreviations

AAA	Abdominal Aortic Aneurysm
AAAP	Aortic Aneurysm Antigenic Protein
ABC	Avidin-Biotin Complex
AOD	Atherosclerotic Occlusive Disease
CD	Cluster Of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CT	Computerised Tomography
Cys	Cystein
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
ECM	Extracellular Matrix
EDP	Elastin Derived Peptide
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMMPRIN	Extracellular MMP Inducer
EVG	Elastin Van Gieson
FCS	Foetal Calf Serum
Glu	Glutamic Acid
Gly	Glycine
H&E	Haemotoxylin and Eosin
His	Histidine
HLA	Human Leukocyte Antigen
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Ile	Isoleucine
IMS	Industrial Methylated Spirit
IMV	Inferior Mesenteric Vein
kDa	Kilo Dalton
LDL	Low Density Lipoprotein
Leu	Leucine
M	Molar
MCP	Monocyte Chemoattractant Protein
MEM	Minimum Essential Medium
MHC	Multiple Histocompatibility Complex
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
MT-MMP	Membrane-Type Matrix Metalloproteinase
PAI-1	Plasminogen Activator Inhibitor-1
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate

SMC	Smooth Muscle Cell
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline-Tween
TGF	Transforming Growth Factor
TIE	TGF Inhibitory Element
TIMP	Tissue Inhibitor of Metalloproteinase
TNF	Tumour Necrosis Factor
tPA	Tissue Plasminogen Activator
TPA	Tumour Promoter Activator
uPA	Urokinase Plasminogen Activator
VCAM	Vascular Cell Adhesion Molecule
VEGF-2	Vascular Endothelial Growth Factor-2
Zn	Zinc

CHAPTER ONE

The Matrix Metalloproteinases

1.1 *Characteristics*

The matrix metalloproteinases (MMPs) are an expanding family of endopeptidases whose primary function is the degradation of extracellular matrix (ECM) components. All members of the MMP family are structurally similar, yet their substrate specificity is variable and exhibits some redundancy.[1] Fundamental characteristics of all MMPs include:

- a dependence on an active site zinc atom for catalysis
- their secretion as latent zymogens which require activation
- their inhibition by tissue inhibitors of metalloproteinases (TIMPs)
- their similar DNA and amino acid sequences
- their ability to degrade at least one component of the ECM

Of the twenty MMPs identified up to 1999 (summarised in Table 1.1), four main subgroups have been determined: collagenases; stromelysins; gelatinases; and membrane type metalloproteinases (MT-MMPs). The collagenases (MMP-1, 8 and 13) cleave the native helix of fibrillar collagen type I, II and III at a single site by cleavage either at Gly_{775}-Ile_{776} or Gly_{775}-Leu_{776} bonds.[2,3] The gelatinases (MMP-2 and 9) degrade types IV, V, VII and X collagens, and efficiently degrade elastin and the gelatin breakdown products of collagenase [4,5] (Figure 1.1). The stromelysins (MMP-3,10 and 11) have very broad substrate specificity's, including laminin and proteoglycans,[6] and also are involved in the activation of other MMPs. MMP-19 has only recently been identified and its biochemical characteristics most closely matched those of the stromelysins.[7]The MT-MMPs (MMP-14, 15, 16, 17) appear to exist primarily as activators of other MMPs and even perhaps as receptors of secreted enzymes.[8] ECM substrate specificity's have not yet been characterised for this subfamily of enzymes, although a collagenolytic and gelatinolytic activity has been described for MT1-MMP.[9,10] For a comprehensive review, see Birkedal-Hansen *et al,* 1993.[11]

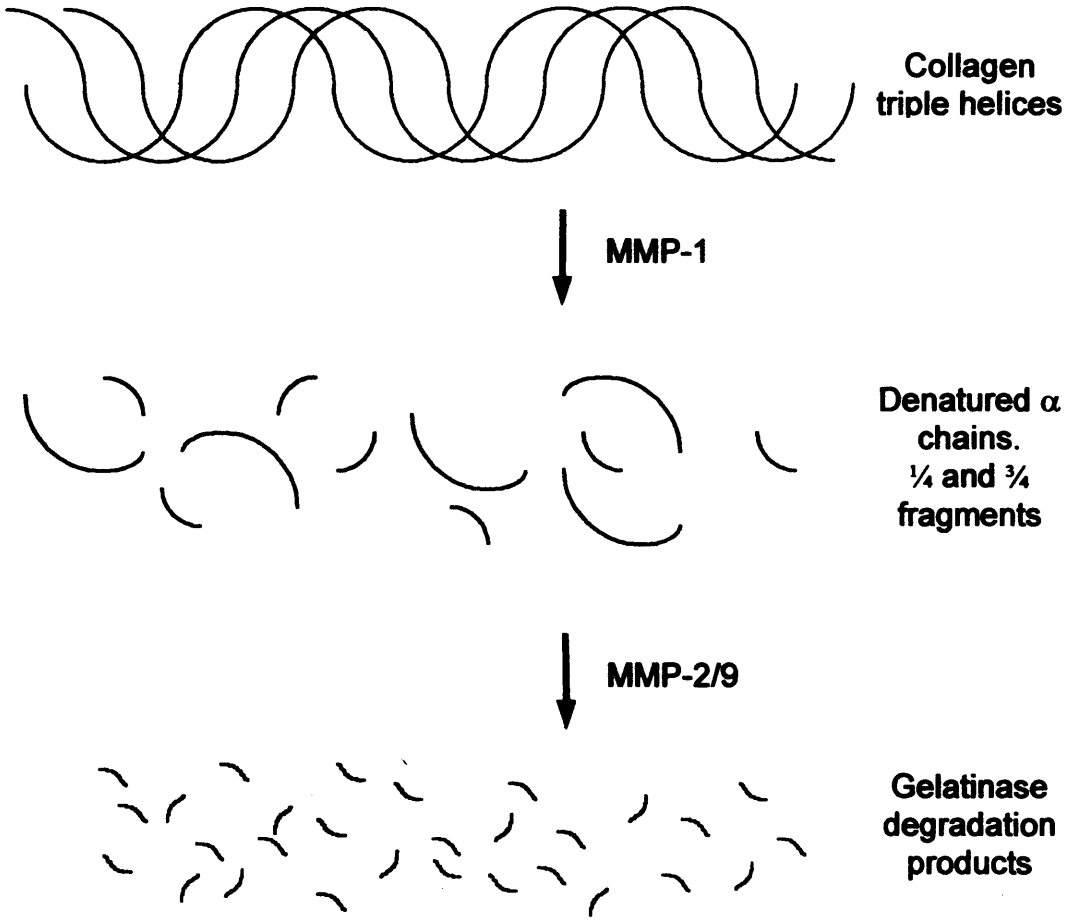
Missing from this sub-grouping system are a number of enzymes. MMP-7 shares some of the structural features of the stromelysins and has a similarly broad substrate spectrum, including proteoglycans and elastin.[12-14] MMP-12 has only relatively recently been identified and appears to degrade many matrix components.[15] MMP-20 is a more recent discovery and is not yet characterised,[16] as is MMP-23.[17,18]

Table 1.1. The Matrix Metalloproteinases.

Enzyme	MMP	Substrate	Latent Size (kDa)
Collagenases			
Interstitial collagenase	MMP-1	Collagen I,II,III,VII,X, gelatin, entactin, aggrecan, link protein	55
Neutrophil collagenase	MMP-8	Collagen I, II, III, aggrecan, link protein	75
Collagenase 3	MMP-13	Collagen I, II, III	
Stromelysins			
Stromleysin 1	MMP-3	Aggrecans, gelatin, fibronectin, laminin, large tenascin c, vitronectin	57
Stromelysin 2	MMP-10	Aggrecan, fibronectin, collagen IV	57
Stromleysin 3	MMP-11	Fibronectin, laminin, collagen IV, aggrecan, gelatin	51
Gelatinases			
Gelatinase A	MMP-2	Gelatin, collagen IV, V, VII, X, fibronectin, laminin, large tenascin C, vitronectin	72
Gelatinase B	MMP-9	Gelatin, collagen IV, V, XIV, aggrecan, elastin, entactin, vitronectin	92
Membrane-Type MMPs			
MT1-MMP	MMP-14	Collagen I+III, fibronectin, laminin, vitronectin, MMP-2, MMP-13	66
MT2-MMP	MMP-15	MMP-2, collagen I+III, fibronectin, laminin, nidogen, tenascin, aggrecan, perlecan	
MT3-MMP	MMP-16	MMP-2, gelatin, collagen III, fibronectin	
MT4-MMP	MMP-17	MMP-2	
MT5-MMP		MMP-2	
Others			
Matrilysin	MMP-7	Aggrecan, fibronectin, laminin, gelatin, collagen IV, elastin, entactin, small tenascin c, vitronectin	28
Metalloelastase	MMP-12	Elastin	57
Collagenase 4 (*Xenopus*)	MMP-18[a]	Collagen I	
Unnamed	MMP-19[a]	Unknown	
Enamelysin	MMP-20	Unknown	
XMMP (*Xenopus*)	MMP-21	Unknown	
CMMP (Chicken)	MMP-22	Unknown	
Unnamed	MMP-23	Unknown	

The MMPs are fundamentally important in the development and maintenance of tissue integrity. They are involved in morphogenesis, wound healing, menstruation and bone resorption. However, pathological states such as rheumatoid arthritis, invasive cancers and vascular disorders are thought to progress as a result of the excessive production of activated MMPs. As potent mediators of ECM catabolism, it is vitally important that MMP production and activation is tightly regulated. The three tier system of MMP control will be discussed in detail later in this chapter.

Figure 1.1 Cleavage of fibrillar collagen helix by collagenases, yielding characteristic fragments which may then be cleaved by gelatinases.



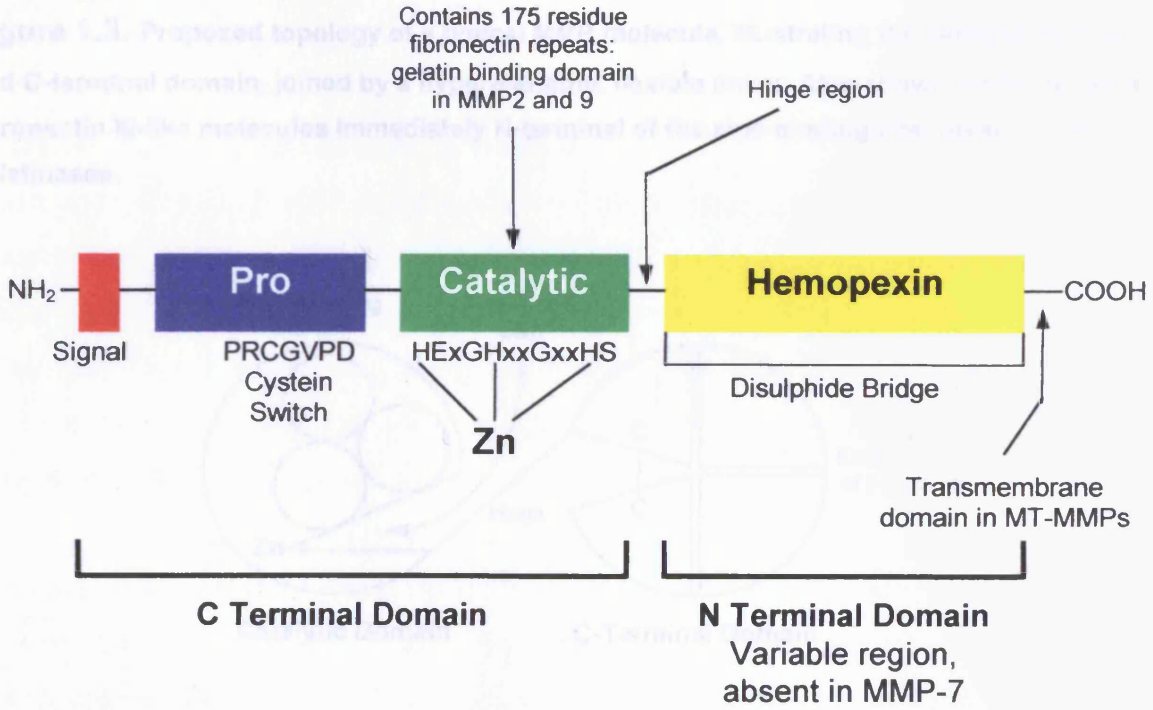
Adapted from Galis *et al.* 1995[19]

1.2 *MMP Structure*

In their unprocessed form, all MMPs may be broadly split into two main parts, the N terminal portion and the C terminal portion. Closer examination allows the definition of several distinct sub-domains, each with specific functions (Figure 1.2). All MMPs have at their N-terminal end a signal peptide to direct their transport to the rough endoplasmic reticulum and the exocytotic pathway. The next domain is a ubiquitous pro-peptide of 77 to 87 amino acids. This domain is cleaved off during activation of the enzyme and plays a fundamental role in the maintenance of latency, as will be described in detail later in this chapter.

The third sub-domain is the major functional portion of the enzyme, termed the catalytic region. A conserved amino acid sequence (His-Glu-X-X-His) facilitates the binding of a zinc atom which is essential for substrate catalysis. The catalytic region is essentially identical in form and function in all MMPs. There are two main modules, separated by a deep active site cleft, with the catalytic zinc atom located at its base[20] (Figure 1.3). In the gelatinases, this domain contains three tandem fibronectin repeats which determines the affinity of these enzymes for gelatin, and are known to confer binding of the enzymes to a variety of ECM components.[21]

Figure 1.2. Diagrammatic representation of the domain structure of matrix metalloproteinases, illustrating the three major sections of a typical MMP.

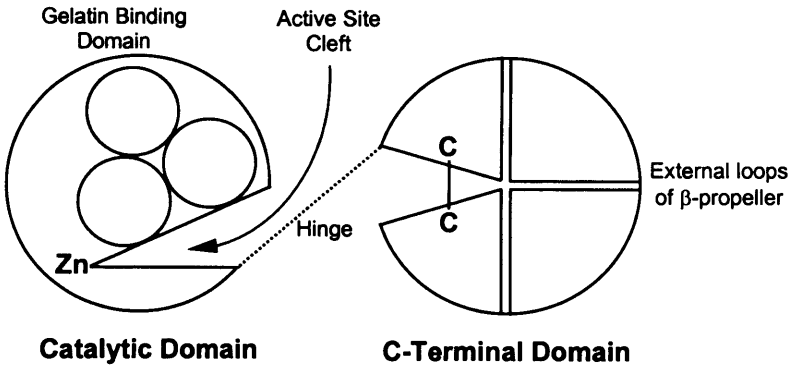


Crystallographic analysis reveals that the N and C terminal domains of MMPs appear to be folded into two quite separate structures. Joining them is a flexible link termed the "hinge" peptide. This is variable in length, ranging from 16 amino acid residues in collagenase (MMP-1) to 65 in MT2-MMP (MMP-15), the latter including 24 prolines. A motif resembling the triple helical collagens is seen in the hinge region of Gelatinase B.[22]

Beyond this point, and up to the C-terminus, is a variable domain which consists of hemopexin-like regions which are thought to be central to substrate specificity and binding.[23] However, MMP-7 does not possess this domain and is considerably smaller than the other MMPs as a result. Crystallography studies show that all MMP C-terminal structures studied so far exhibit that same four bladed β-propeller topology, made of antiparallel, four stranded β sheets arranged around a tunnel.[24] A disulphide bridge links cystein residues on the first and fourth blades, a feature of all MMPs and important in the function of this domain.[24-27]

Biochemically, the C-terminal domain fulfils a number of roles in MMP function. As mentioned, in the collagenases this domain mediates binding of the enzyme to interstitial collagens.[28-31] Uniquely, at their C-terminus end, the MT-MMPs possess a transmembrane domain, by which they locate at the extracellular surface.

Figure 1.3. Proposed topology of a typical MMP molecule, illustrating the catalytic domain and C-terminal domain, joined by a hyper-variable, flexible linker. Also shown are three tandem, fibronectin III-like molecules immediately N-terminal of the zinc-binding site, present only in the gelatinases.



1.3 *Control Of MMP Activity*

1.3.1 *The Three Phase Regulation Of MMPs*

MMPs are critical components in the genesis, remodelling and repair of all tissues. Their capacity for degradation of ECM and basement membrane components aids the removal of aged or damaged tissue, so that it may be replaced by newly synthesised structural elements. However, it is this collective ability of the enzymes to break down the ECM that poses a significant threat to tissue integrity if not tightly controlled.

Regulation of MMP action is exerted at three separate levels. Transcriptional control of the majority of MMPs ensures that the enzymes are produced only in response to specific signalling events. Once expressed, two further mechanisms operate to prevent enzyme activity occurring: firstly the enzymes are produced as latent zymogens which require a processing event to become activated. Secondly, catalysis by active enzymes is prevented by binding to the TIMPs.

1.3.2 *Gene Structure And Control Of Transcription*

Although regulation of MMP expression has not been widely studied, it is clear that there are complex and individualised patterns of expression for the various members of the family. Under normal physiological circumstances, the majority of the MMP genes are not transcribed. Only in remodelling tissue, such as the endometrium during the menstrual cycle, are many of the MMP genes seen to be induced, and in general their overproduction is associated with pathological states.

Of all the MMPs, the most widely detected is MMP-2 (gelatinase A), which is normally expressed by most mesenchymally derived tissues and many other normal adult tissues.[32] Others, such as MMP-1, 3 and 9 are produced by macrophages and vascular smooth muscle cells, but in general do so only when stimulated.[33]

The patterns of expression of the various MMPs are reflected in the regulatory elements upstream and within the MMP genes.[34] Analysis of the promoter regions reveals common sites for the binding of specific transcription factors, and provides insight into their functional relevance.

The MMP-2 gene promoter contains regions characteristic of constitutively expressed or "housekeeping" genes, and explains in part the widespread expression of this enzyme.[35] In contrast to most other known MMP gene promoters, MMP-2 lacks a TATA box and a TPA-responsive (fos/jun) element (TGAGTCAG), the latter a binding site for the transcription factor AP-1.[36] Other unusual features include two sp1 binding sites (GGGCGG) in the promoter and one AP-2 site (CCCCAGGC) in the first exon.

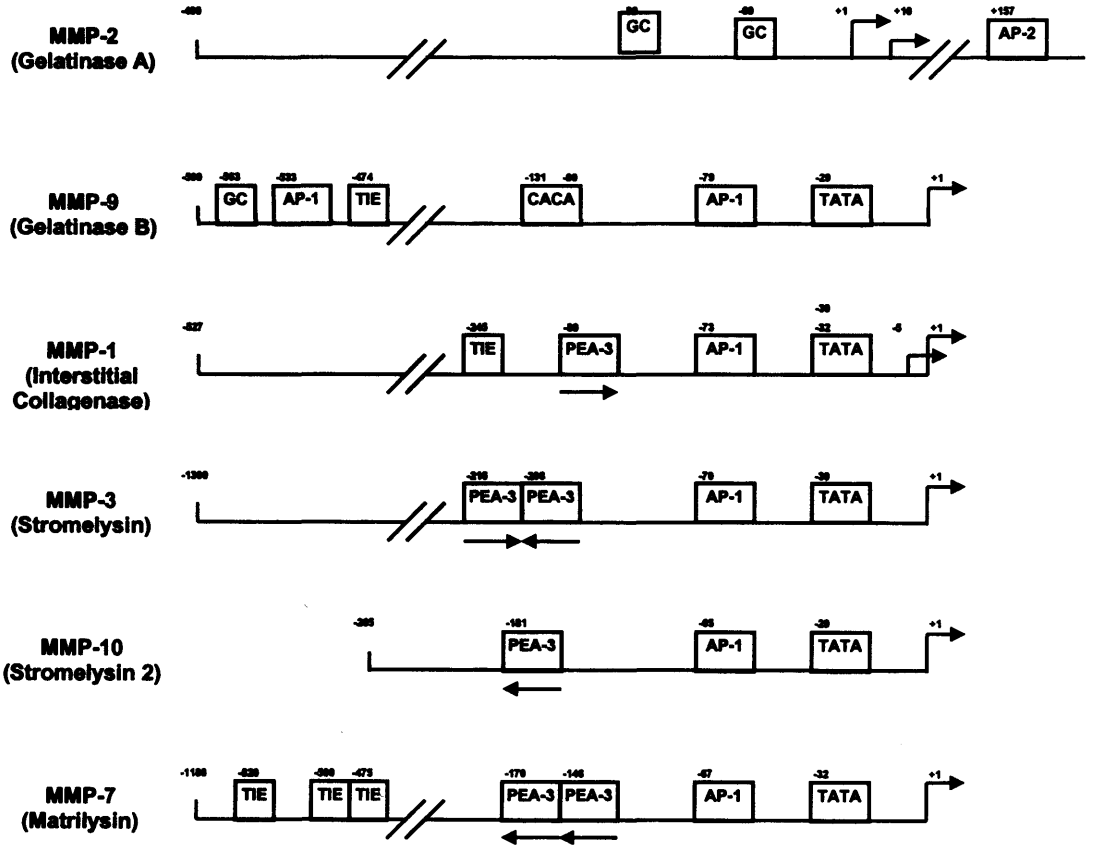
These observations contrast strongly with the promoters of MMP-1, 3, 7, 9 and 10,[22,37-39] all of which contain an AP-1 binding site at about -70 and a TATA box at about -30. Other elements, such as PEA-3 binding motifs in MMP-1, 3 , 7 and 10, or TGF-β inhibitory elements (TIE) in MMP-1, 7, and 9, characterise these genes as being highly controllable.[34] The AP-1 site binds a protein complex made up of products of the *fos* and *jun* oncogene families and is essential for the induction of expression of the MMP-1 and MMP-3 genes.[40-42] PEA-3 elements bind transcription factors of the c-*ets* family[43] and the AP-1 and PEA-3 sites together are termed the oncogene response or tumour promoter element. This combination of transcription factor binding sites is responsible for growth factor and oncogene mediated expression of MMP-1[44] and MMP-3.[42]

A number of groups have examined the inhibition of MMP gene expression, for example dexamethasone inhibition of MMP-1,[45,46] retinoic acid inhibition of rat MMP-3[47] and TGF-β inhibition of rat MMP-3.[48] With dexamethasone and retinoic acid, the steroid and receptor appeared to complex together with or interfere with AP-1 mediated expression, repressing transcription of the gene.[45-47] In rats, TGF-β interacted with a specific TIE element which was essential for the inhibitory effect.[48] This interaction may also involve c-FOS, complicating the understanding of positive and negative regulation of gene expression. Figure 1.4 summarises the promoter regions discussed.

The MMP genes themselves exhibit highly conserved, modular structure, as represented in Figure 1.5. The basic MMP prototype is that of MMP-1,[49] and variations on this are thought to have given rise to the other family members. This homology is reflected in the fact that at least some of the MMPs are co-ordinately regulated, particularly with regard to AP-1 and PEA-3 transcription factor binding events. However, selective expression of specific MMP genes may still occur without concomitant effects on others, and it is readily apparent that

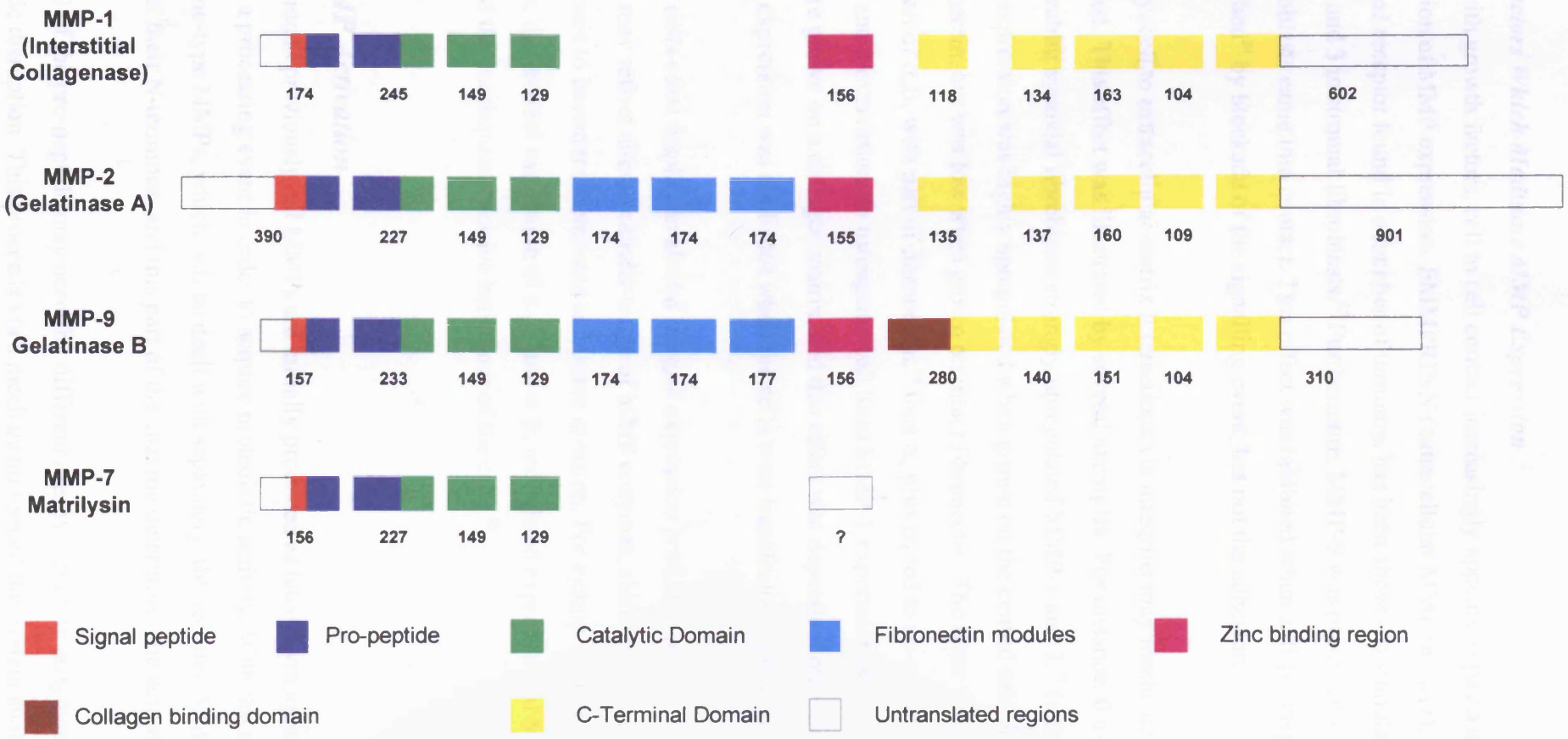
whilst AP-1 in particular appears to be important for transcription, other elements are crucial to the level and specificity of gene expression.[36,42,50] The complexity of this regulatory apparatus is a further indication of the necessity for tight control over MMP production.

Figure 1.4 Human MMP gene promoter regions.



Adapted from Matrisian LM. 1994.[51]

Figure 1.5 Examples of the modular structures and products of human MMP gene exons.



Adapted from Matrisian L.M. 1992.[52]

1.3.3 *Factors Which Modulate MMP Expression*

Along with growth factors, cell to cell contact increasingly appears to pay a major part in the modulation of MMP expression. EMMPRINN (extracellular MMP inducer), a membrane-associated receptor found in a number of tumours, has been shown to stimulate release of MMP-1 and 3 in stromal fibroblasts.[53] Furthermore, MMP-9 was produced when tumour cells and fibroblasts came into contact. This effect was inhibited when anti-β_1 integrin antibody was applied[54] by blockade of the signalling event, but not the adhesion.

Similarly, cell to extracellular matrix interactions via integrins may modulate MMP expression. This effect was illustrated by several examples. For instance, the cross-linking of $\alpha_5\beta_1$ on rabbit synovial fibroblasts strongly upregulated MMP-1 and 3.[55] In the same cell system, expression was highly upregulated when grown on the central cell-binding fragment of fibronectin, but was low when grown on intact fibronectin. The latter was dependent on interaction of $\alpha_4\beta_1$ with part of fibronectin,[56] thus α_4 plus α_5 led to co-operative control of MMP-1 and 3 expression. In osteogenic cell lines MMP-1 expression was upregulated when cells were grown on a collagen matrix, and this effect was dependent on expression of $\alpha_2\beta_1$. MMP-1 expression was abolished when the cells were transfected with α_2 antisense cDNA.[57]

Tumour cells often display an altered integrin expression profile. It is now being proposed that this may reflect altered cellular usage of MMP enzymes, shifting away from ECM homeostasis to basement membrane and tissue invasion. For example, in human melanoma cell lines, differential expression of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ modulated expression of MMP-2 and promoted the subsequent invasive behaviour of the cells.[58]

1.4 *MMP Activation*

As mentioned previously, all MMPs are initially produced as latent zymogens which must undergo a processing event in order to acquire proteolytic activity. With the exception of the membrane-type MMPs, which will be dealt with separately, the secreted MMPs include a pro-peptide at their N-terminus, and this part of the enzyme determines the activity status.

Removal of the pro-peptide may occur by different means, either by enzymatic or non-enzymatic disruption. This proceeds via a mechanism termed the cystein switch, first

described by Van Wart and colleagues.[59] In simplified terms, the cystein switch refers to the interaction of the active site zinc atom and an unpaired thiol group of a cystein residue located within a conserved region of the pro-peptide (PRCGVPD). Activation of the enzyme begins with the disruption of this bond (the switch open state), and causes conversion of the zinc atom to a catalytic state by conformational change. A series of processing events, including autolytic cleavage, brings about complete activation of the enzyme, associated with a reduction in size and molecular weight.

1.4.1 *The Maintenance Of MMP Latency*

The main feature in the maintenance of MMP latency is the bond between the active site zinc atom and the cystein residue located within a conserved region of the pro-peptide. Cleavage of this bond may be brought about by either proteolytic or non-proteolytic means. In both cases, the Zn is allowed to associate with a water molecule, which is required for catalysis. Disruption of the bond by proteinase attack is brought about by direct cleavage of a part of the propeptide, the so-called "bait" region. This area is particularly susceptible to proteinase attack, and when cleaved causes a conformational change in the propeptide which breaks the Zn-Cys interaction, rendering the propeptide more readily cleaved from the enzyme. This latter cleavage is most often catalysed by an MMP and is commonly autolytic. As a general rule it is not made by the proteinase which made the original attack.[60]

For example, plasma kallikrein activation of MMP-1 was shown to occur by cleavage of the pro-peptide at residues 35-36, opening the switch, probably due to the instability of the resultant structure.[60] This was followed by a series of autoproteolytic cleavages, including one at residue 64 in the pro-peptide, to generate the fully processed and active form.[60-62] Such events were observed in most of the secreted MMPs, but with minor alterations according to the mediator of the initial cleavage. Table 1.2 illustrates the common activating proteases and the mediators of secondary cleavage events which generate fully active MMPs.

Table 1.2. Mediators of the activation of MMPs.

Zymogen	Activation By	
	Initial Step	Final Step
ProMMP-1	Trypsin, plasmin, plasma kallikrein	MMP-2, MMP-3, MMP-7, MMP-10, Chymase[a]
ProMMP-2	MMP-14, MMP-16, MMP-1	MMP-2, MMP-7
ProMMP-3	Many proteases, but not MMPs e.g. trypsin, chymotrypsin, plasmin, chymase, leukocyte elastase, pseudolysin, thermolysin	MMP-3[b]
ProMMP-7	Trypsin, plasmin, leukocyte elastase	MMP-3[b], MMP-7
ProMMP-8	Tissue kallikrein, leukocyte elastase, cathepsin G, trypsin	MMP-3[b], MMP-10
ProMMP-9	MMP-1, MMP-2, MMP-3, MMP-7	MMP-1, MMP-2, MMP-3, MMP-7
ProMMP-10	Plasmin, trypsin, chymotrypsin	MMP-10
ProMMP-11		Furin[b]
ProMMP-13	MMP-3, MMP-14	MMP-3, MMP-13, MMP-2
ProMMP-14		Furin[b]

[a] Only partial activity is detected. [b] Direct activation

Adapted from Nagase, H, 1997.[63]

1.4.2 *Serine Protease And Other Mediators Of MMP Activation.*

As may be seen in Table 1.2, plasmin is a common activator of MMPs. Plasmin is important in the fibrinolytic system, which consists of a proenzyme, plasminogen, which may be activated either by tissue plasminogen activator (tPA) or by urokinase-like plasminogen activator (uPA), causing conversion to plasmin. tPA-mediated activation is mainly involved in the blood clotting cascade, specifically in the dissolution of fibrin in the circulation.[64] In contrast, uPA binds to a cell surface receptor, uPAR and the uPA/uPAR complex mediates localised activation of plasminogen, being associated with pericellular proteolysis.[65] Inhibition of the fibrinolytic system may occur either by blockage of plasminogen activation by plasminogen activator inhibitor-1 (PAI-1) or by blocking the activation product, plasmin, by α_2 antiplasmin.[64]

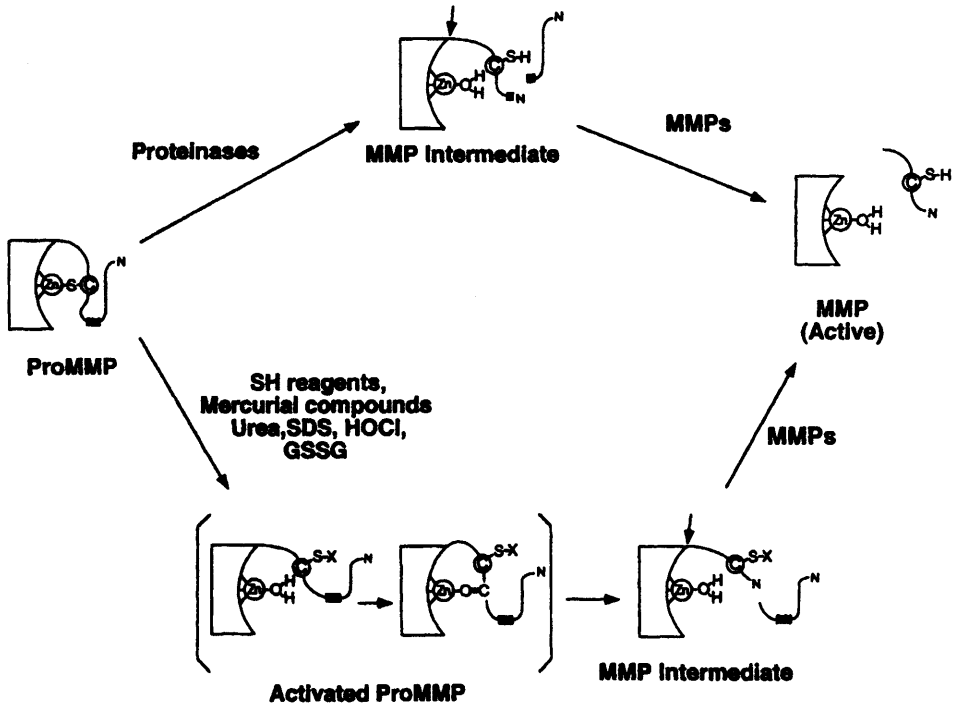
Plasmin is known to be a potent activator of many MMPs.[52,66,67] MMP-2 is not activated by plasmin but may be activated directly by uPA.[68] Thrombin, the immediate precursor of

fibrinogen in the blood clotting cascade,[69] has also been shown to be capable of activating SMC-derived MMP-2.[70]

The potential for the initiation of a proteolytic cascade, with active MMPs activating others, demonstrates the potential importance of plasmin in ECM remodelling, particularly with respect to disease. This fact is supported by evidence that plasmin is readily detected in various pathological states associated with ECM degradation, such as vascular stenosis.[71] Similarly, high levels of plasminogen activators may be found in such tissues.[72]

MMPs are susceptible to other, chemical modifications. Organomercurials, thiol reagents, metal ions and oxidants may cleave the pro-peptide and disrupt the Zn-Cys interaction, shifting the switch equilibrium from closed to open. For example, reaction of MMP-1 with 4-amino phenyl mercuric acetate caused the initial disruption and was followed by two autoproteolytic cleavages within the pro-peptide at residues 67-68 and 82-83. Following this event(s) the pro-peptide was completely cleaved and the active site exposed (Figure 1.6). This pattern of activation is common to the majority of secreted MMPs. The stepwise pathway of bond cleavage, conformational change, intermediate formation, autoproteolytic removal of propeptide to produce active enzyme is exhibited by all MMPs except the MT-MMPs.[63]

Figure 1.6. Cleavage events leading to MMP activation, illustrating the two pathways to enzyme activity. The top pathway represents proteinase cleavage of the propeptide, whilst the bottom pathway indicates the events produced by chemical modification.



1.4.3 *Membrane Type Metalloproteinases*

The membrane-type metalloproteinases (MT-MMPs) represent a fundamentally distinct sub-grouping within the MMP family. Their structure is familiar, containing the typical N-terminal domains of pro-peptide and catalytic regions. However, beyond the typical C-terminal domain resides a transmembrane motif, such that the protease may locate at the extracellular surface. Furthermore, the five MT-MMPs undergo a different activation pathway to the secreted enzymes, being activated intracellularly and emerging at the cell membrane in an active state. For a review, see Seiki, 1998.[73]

The first MT-MMP identified was isolated by Sato and co-workers as a membrane-associated activator of MMP-2.[74] Since the cloning of MT1-MMP, four further MT-MMPs have been isolated, all of which activate proMMP-2.[75,76] Initial studies appear to suggest that the primary function of the MT-MMPs is as activators of secreted MMPs, and in the case of MMP-2, as a cell surface receptor to localise proteolytic potential.

As mentioned, the MT-MMPs are activated intracellularly, being cleaved at a conserved site by the golgi-associated protease, furin. The furin recognition motif was first identified in MMP-11 (stromelysin-3),[9] which is also activated in this manner and is unique amongst the secreted MMPs. The furin-mediated intracellular activation of MT1-MMP has since been confirmed,[77] and it was proposed that the identification of furin recognition motifs in all four MT-MMPs indicates a probable common activation pathway.

The mechanism by which MT1-MMP activates MMP-2 is probably the most widely studied, but has not yet been clearly defined.[63] It was demonstrated that the membrane-associated enzyme would bind and activate proMMP-2, and that this reaction may be enhanced by the addition of TIMP-2. However, this occurred up to a maximum level, beyond which the addition of further TIMP-2 inhibited activation. Moreover, an anti-TIMP-2 antibody blocked activation of MMP-2 by MT1-MMP, suggesting that a three way interaction between the enzymes and inhibitor was fundamental to this process.

Recent reports indicated that MT1-MMP was also involved in the activation of MMP-13 (procollagenase 3), and that this reaction was accelerated by the presence of MMP-2. MT1-MMP has also been shown to degrade a number of extracellular matrix components, such as collagens I, II and III,[10] fibronectin, laminin-1 and vitronectin.[9]

1.5 *Tissue Inhibitors Of Metalloproteinases (TIMPs)*

1.5.1 *Definition*

The TIMPs are specific inhibitors of the matrix metalloproteinase family of enzymes. After control of gene expression and the necessity for activation of the latent MMPs, the TIMPs represent the third and final level of control of enzyme activity *in vivo*. TIMPs form strong, specific, non-covalent interactions with MMPs with a stoichiometry of 1:1 and K_i values of less than 10^{-9}M.[78]

To date (October 1999), four members of the TIMP family have been described.[79-82] TIMP-1 and TIMP-2 are found in many body tissues and fluids, and may be detected in the medium of many cultured cell lines. TIMP-3 appears to be associated with the extracellular matrix, and is relatively insoluble. Its inhibitory profile is markedly similar to TIMP-1 and 2.[83] TIMP-4

shares homology with TIMP-2 and evidence appears to suggest a similar inhibitory profile to TIMP-2, albeit with reduced effect.[82] Refer to Table 1.3.

Table 1.3 The tissue inhibitors of metalloproteinases.

	Size (kDa)	Amino Acid Residues	Gycosylation State
TIMP-1	28.5	184	Glycosylated
TIMP-2	21	194	Non-glycosylated
TIMP-3	21	218	Non-glycosylated
TIMP-4	23	195	Non-glycosylated

1.5.2 *Structure*

Structurally the TIMPs are very similar. Their DNA sequence homology is around 40% and their protein homology approximately 25%, but their molecular structures are identical. Most importantly, twelve cystein residues are conserved across the TIMP family, which form six fundamental disulphide bonds.[84] These bonds maintain the tertiary structure of the TIMP molecule, producing a very stable, six-looped form[85] (Figure 1.7).

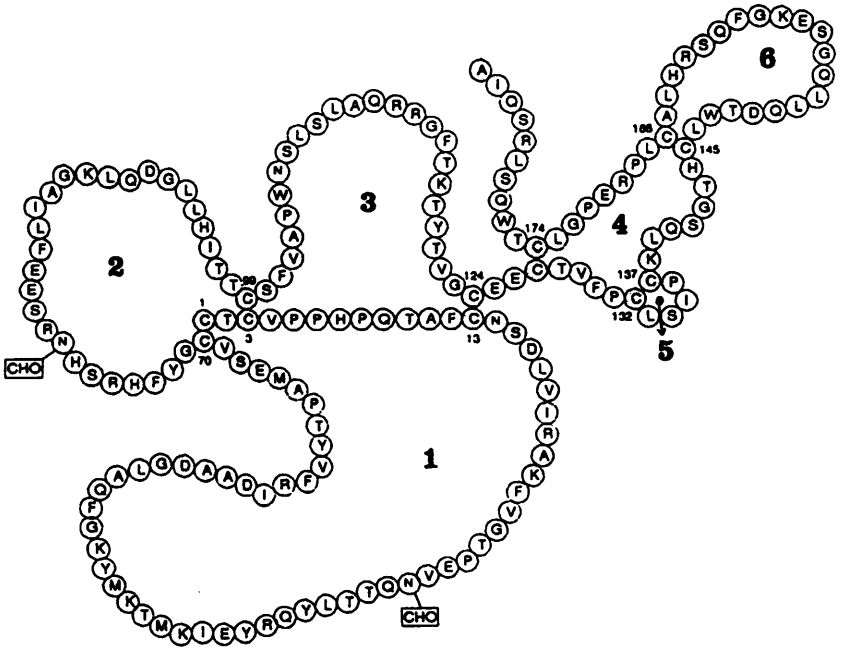
The TIMP molecule, like the MMPs, is split into an N-terminal and a C-terminal domain. The N-terminal domain consists of loops 1-3, and alone may act as a functional MMP inhibitor.[86,87] This region binds to the N-terminal catalytic domain of the MMPs and interacts with the active site zinc atom. The C-terminal domain consist of loops 4-6, and contains at least two binding sites which interact with MMPs and affect the level of inhibition according to the enzyme involved.[88]

1.5.3 *Model Of Binding Between TIMPs And MMPs*

TIMP-1 interacts most strongly with MMP-1, 3 and 9, whereas TIMP-2 interacts with MMP-2. In one of the best studied examples of MMP-TIMP binding, the specific N-terminal region of TIMP-1 which interacts with the MMP-9 active site was shown to be the second disulphide knot.[89] This is the area where the N and C terminals of the TIMP molecule meet and is bounded by amino acids Cys_{13}-Cys_{124} and Cys_{127}-Cys_{174}. A similar interaction is thought to occur between TIMP-1 and MMP-1.[90] Mutation analysis has shown the necessity for C-terminal MMP-TIMP interaction in conjunction with the N-terminal inhibitory action. In combination, the association rate constants are greater than for either terminal alone,

suggesting that the C-terminal associations help to align the N-terminal inhibitory motif of the TIMP and the enzyme's active site.[88]

Figure 1.7 The six-looped structure of the TIMP molecule, illustrating the twelve cystein residues (© numbered) important in maintaining tertiary structure and the N and C terminal domains, delineated by loops 1-3 and 4-6 (numbered in bold) respectively.



Unexpectedly, it is still possible for the MMPs to be activated when in complex with the TIMPs. In fact, the concentration of TIMP appears to play a role in determining the activation status of the enzyme. The TIMP-2/MMP-2 complex may undergo activation by organomercurials, but the specific activity is only 5-10% of that of uninhibited enzyme. This enzyme may be further inhibited by the addition of more TIMP-2, suggesting that there may be more than one TIMP binding site on the MMP molecule.[91] Furthermore, it has been shown that TIMP-2 retains inhibitory activity for MMP-1, even when already in complex with MMP-2, suggesting that TIMP-2 may also have more than one binding site for MMPs.[92]

The role of TIMP in MMP activation is therefore more complex than was at first appreciated. Another example of this was the involvement of TIMP-2 in the MT1-MMP-mediated activation of MMP-2. Low levels of TIMP-2 added to HT1080 tumour cell membrane preparations promoted activation of MMP-2, but at higher concentrations MMP-2 activation was inhibited.[93] The authors of this work proposed that this effect was due to the interaction of

TIMP-2 with MT1-MMP, the membrane bound activator of MMP-2 *in vivo*. At low concentrations they suggested that MT1-MMP receptors were available for the binding and activation of MMP-2, but at high TIMP-2 concentrations, all MT1-MMP was inhibited.

The credibility of this theory of three way interaction was enhanced by evidence that the TIMP-2 molecule interacts with the MT1-MMP molecule via its N terminal, leaving its C terminal free to interact with MMP-2.[88,93] Evidence for interactions between other TIMPs and MT-MMPs is not yet available, but the conservation of sequences across the two subfamilies suggests that this may well occur.

CHAPTER TWO

Abdominal Aortic Aneurysm

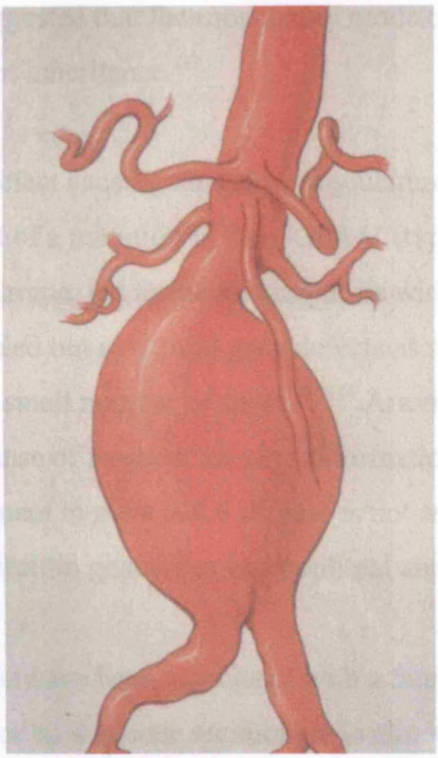
2.1 *Introduction*

An aneurysm is a permanent, localised dilatation of an artery, resulting from a loss of structural integrity. The term aneurysm is derived from the Greek *aneurynein*, meaning to widen or dilate. The most widely accepted definition is that given by the Society for Vascular Surgery and the International Society of Cardiovascular Surgery, which states that "an aneurysm can be defined as a permanent localised dilatation of an artery having at least a 50% increase in diameter of the artery in question."[94]

Research in the last decade has demonstrated that aneurysms are not easily understood. Recent advances have revealed the complex, dynamic nature of the wall of the aneurysmal vessel, a region of vigorous tissue remodelling and cellular activity. Far from being a simple, balloon-like swelling, the aneurysmal vessel has come to be seen as a multi-faceted disease state, encompassing excess proteolysis and synthesis of extracellular matrix components, cellular migration and death, the involvement of the immune system and the influence of predisposing factors both environmental and genetic.[95]

Aneurysms occur at many sites and may take different forms. A number of classification systems exist, based generally on these criteria. For example, the site may be cerebral or popliteal, and they may exist in the saccular or fusiform state. Figure 2.1 illustrates a typical fusiform aortic aneurysm. Furthermore, aneurysms are seen to develop as a result of specific causative factors, such as Marfan and Ehlers Danlos syndromes, infection, blunt trauma or inflammation.[94] However, the most common aneurysm is that of the abdominal, infra-renal aorta, normally termed the non-specific or atherosclerotic aneurysm. The abdominal aortic aneurysm (AAA) will be the subject of this thesis, in particular the initiation of aneurysmal disease.

Figure 2.1. A typical fusiform abdominal aortic aneurysm.



2.2 *Epidemiology*

Abdominal aortic aneurysms occur predominantly in elderly males, who are affected four times as often as females,[96] and whites three times as often as blacks.[97] Recent studies indicate that the prevalence of AAA >3cm in diameter in males over 65 years is approximately 7%[96,98] and that there has been an increase in incidence over the past decade. This has been demonstrated separately in the UK,[98] the USA,[99] Australia[100,101] and Sweden.[102,103]

Various reasons for this increase are proposed. It is most often suggested that the increase in the elderly population, increased awareness and improvement in diagnostic techniques have led to an increased prevalence.[104,105] However, the increase in incidence of AAA since the 1950's is larger than can be explained by these factors alone.[98]

Risk factors predisposing to AAA are both congenital and acquired. Familial clustering of aneurysms has led to speculation on the possible inheritance of the condition.[106-109] Men with an affected first degree relative have a ten-fold greater risk of developing an aneurysm,[109,110] although the mode of inheritance has not been clarified.[111] Intriguingly, AAAs have been

reported in identical twins,[112] reinforcing the suggestion of a strong genetic element to the pathogenesis of the disease. In the most comprehensive study to date, the analysis of 324 probands with aneurysms suggested that the most likely mode of inheritance was as a single gene defect showing dominant inheritance.[113]

The notion of a single gene defect causing abnormal vasculature has been investigated in some depth. The involvement of a mutation in the *COL3A1* (type III collagen) gene in aortic disease in Ehlers Danlos syndrome, led to speculation of its wider involvement in aneurysms, but mutation screening has ruled out structural gene defects as significant factors in AAA formation,[114] in all but a very small number of cases.[115,116] Arterial fibrillin, abnormal in Marfans syndrome and the cause of frequent aneurysm formation,[117] has also been subject to investigation, but its involvement in most AAA disease is not supported. However, there is an association between certain fibrillin genotypes and popliteal aneurysm.[118]

Between 13 and 17% of AAAs have been associated with a familial contribution not due to other inherited disorders,[119] and so a genetic component is almost certainly present in aneurysmal disease. In order to unmask this disease potential, evidence suggests that additional acquired risk factors such as smoking or hypertension are required. Cigarette smoking in particular has received much attention,[120-124] as metabolic products of tobacco smoke inhalation are known to modulate vascular tone by several means, for example by increasing nitric oxide production, which increases vasodilation, influencing SMC proliferation and endothelial permeability.[123] In a separate study, nicotine was shown to affect collagen and collagenase gene expression in cardiac fibroblasts.[125] Hypertension is similarly implicated, as is hypercholesterolaemia, though with less conviction.[120,121,126-128]

Theoretical links with atherosclerosis remain vague and controversial, although it is clearly unsupported as a single causative factor.[129] Aneurysms are seen to develop at the sites normally associated with atherosclerotic, occlusive disease. This, according to some, is compelling evidence that the two conditions are linked. A number of researchers believe that aneurysms occur because of atherosclerotic changes,[130,131] whilst others are convinced that their aetiologies are distinct and independent.[132-134]

It is certain that the aetiology of AAA involves the complex biochemical interaction of environmental and genetic elements, the outcome being the increased likelihood of localised

degenerative change in the aortic wall. In addition to this, the effects of ageing on the vascular system make aneurysm formation complex in the extreme.

2.3 *Treatment*

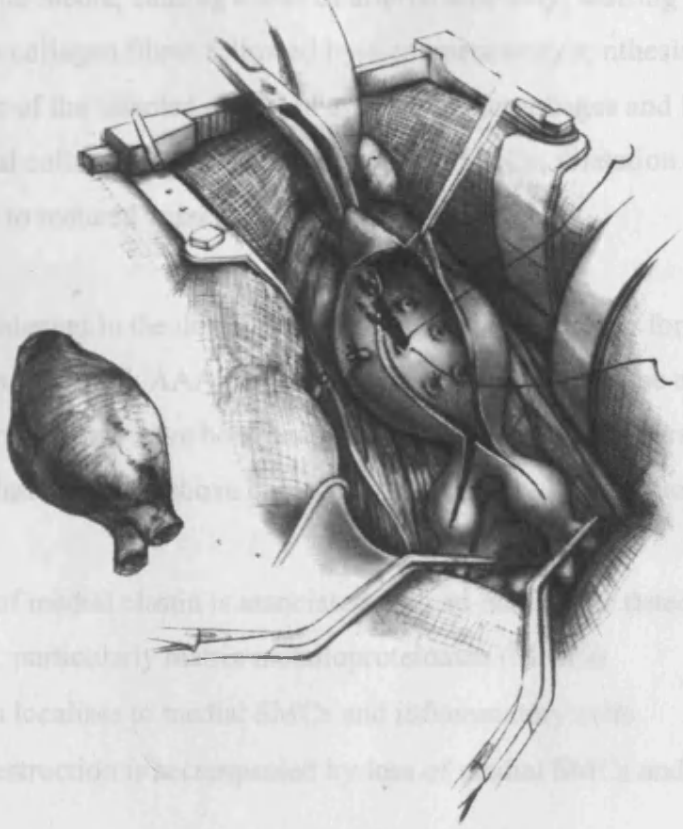
Aortic aneurysms are generally difficult to detect, 38% being found upon general physical examination[135,136] and 31% by radiographic study performed for some other reason.[135] Small aneurysms are generally asymptomatic, but as they enlarge, may manifest as abdominal and/or back pain.[135] The aneurysm must be of approximately 5cm in diameter to be detected by physical examination,[137] and its suspected presence must be confirmed either by ultrasonography, CT scanning or magnetic resonance imaging.[138-140]

Wherever possible, vascular surgeons recommend surgical repair of aneurysms greater than 5.5cm in diameter to avoid future rupture.[141,142] Up to 60% of ruptured aneurysms are immediately fatal,[143] and this figure rises to 90% when combined with the deaths occurring during emergency surgery.[98,144] Ruptured aneurysms account for around 15000 deaths per annum in the US,[145] yet the mortality rate for elective repair surgery is only around 5%.

In order to repair the region of aneurysmal expansion, a graft must be inlayed within the sac to exclude the aneurysm from the arterial circulation. The first such case was performed in 1952,[146] and the technique has evolved since, from the use of human donor artery to replace the affected region, to the current practice of inserting a prosthetic graft. The operation involves an abdominal incision, manipulation of the bowel and dissection of the peritoneal viscera, combined with aortic clamping (Figure 2.2). These factors can lead to pulmonary and gastrointestinal complications and cardiac dysfunction following the procedure.[147]

More recently, endovascular repair of the aortic aneurysm has become feasible. In this approach a graft is inserted into the aneurysm via a remote arterial site, usually the femoral artery, over a guide wire. This is a potentially less invasive and hence less traumatic procedure. The graft is anchored in place by self/balloon-expanded metallic stents.[148] Although avoiding the pitfalls of invasive repair, endovascular techniques suffer from their own drawbacks, which are now becoming more widely understood.[149]

Figure 2.2. The conventional surgical repair of abdominal aortic aneurysm, illustrating the opening of the sac, prior to the insertion of a prosthetic graft to replace the diseased vessel. Reproduced from Bergan and Yao, 1979.[150]



Clearly, some form of early aneurysm detection would be an advantage, but economic considerations have prevented screening programmes from becoming widespread. However, as the disease becomes characterised biochemically, it may eventually be possible to identify molecular markers that could be used as a diagnostic tool. As will later be explained, it has become apparent that aneurysms develop as a result of a combination of genetic and environmental factors. This lends weight to the theory that a genetic marker may be identifiable, and hence a therapeutic strategy devised.

2.4 *Pathogenesis Of Abdominal Aortic Aneurysm*

The AAA has been the subject of intense scientific scrutiny since 1980, when Bussutil and colleagues first reported an increase in collagenolytic activity within the wall of the

aneurysmal aorta.[151] Since that time, a picture has emerged of a sequence of events which may be seen as sub-stages of aneurysm formation and progression. Experimental evidence suggests that aneurysm formation may involve the following events: damage to the elastin fibre network of the arterial media, causing a loss of arterial elasticity; shifting of the pressure-bearing burden onto collagen fibres followed by a compensatory synthesis of adventitial collagen; infiltration of the affected region of artery by macrophages and lymphocytes; destruction of medial collagen fibres and loss of medial SMCs; dilatation of the affected region of artery due to reduced vessel wall integrity.

Due to difficulties inherent in the detection of aneurysms, a time-scale for these events is impossible to assign. Although AAAs are predominantly manifest in the elderly, it is feasible that arterial degeneration may have been occurring over a number of years. Amongst the wealth of data that has led to the above chronology, a few salient points are identifiable:

- The destruction of medial elastin is associated with an increase in detectable elastolytic protease activity, particularly matrix metalloproteinases (MMPs).
- MMP expression localises to medial SMCs and inflammatory cells.
- Medial elastin destruction is accompanied by loss of medial SMCs and infiltration by macrophages.
- The inflammatory cell infiltrate is associated with a loss of medial collagen and thus vessel expansion.
- Expansion is associated with an increase in adventitial collagen production and the number of vasa vasorum.

From these points, which will be discussed in detail in the following sections, it is apparent that damage to medial elastin constitutes the early stage of the disease process. How and why this occurs will be considered by this thesis.

2.5 *Morphology Of The Aorta And Aneurysmal Changes*

Arteries are relatively thick walled vessels, designed to carry blood away from the heart and into a complex vascular system of gradually decreasing diameter, to be distributed amongst body tissues and organs. The pressure necessary to achieve this is very high immediately

downstream from the heart, and consequently the larger vessels must, by virtue of their structural components, resist and recoil against the pulsatile flow of blood passing through them.

The best example of this model of elastic arteries is the aorta, the largest human artery, which leads directly from the heart to all other vasculature except the pulmonary system. The normal human abdominal aorta is approximately 2cm in diameter. Descending from the thorax, the aorta enters the abdominal cavity through the diaphragm. A number of branches emanate from the abdominal aorta, feeding various local organs and tissues, such as the renal arteries, mesenteric arteries and lumbar arteries. At its termination, the aorta bifurcates to become the two common iliac arteries (Figure 2.3).

The infrarenal aorta is the most common site for AAAs. Externally, the aneurysmal aorta is observed as a localised dilatation, but when opened longitudinally, reveals extensive changes in vessel structure. The wall appears thickened, and often contains lesions closely resembling atherosclerosis (Figure 2.4).

Figure 2.3. Anatomy of the human abdominal vascular system. Reproduced from Gosling *et al*, 1990.[152]

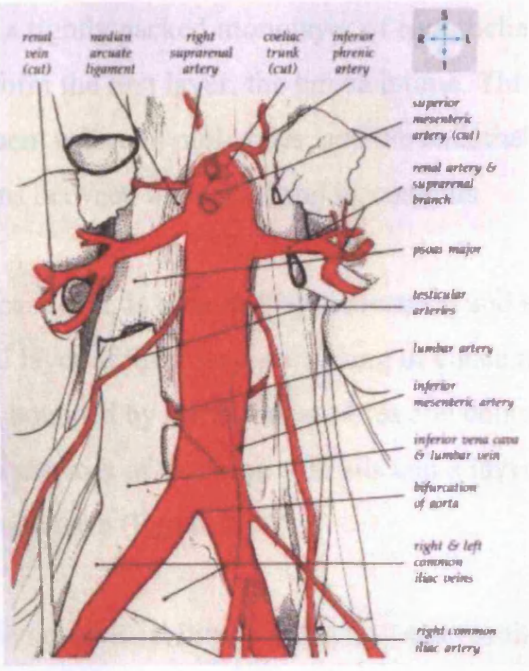
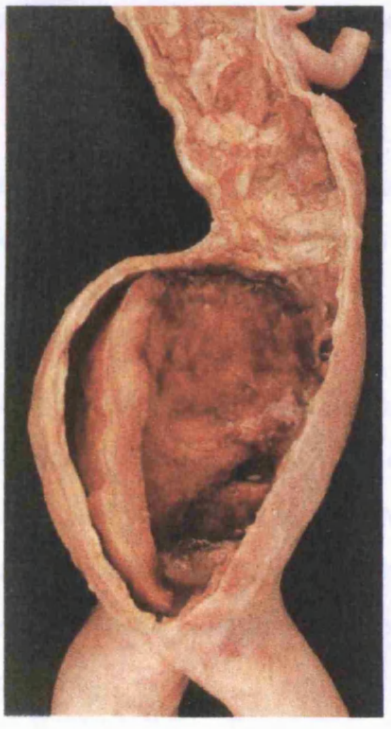


Figure 2.4. Longitudinal section of the abdominal aorta, with obvious aneurysmal dilatation, illustrating the thickened and atherosclerotic vessel wall. Reproduced from Fletcher and McKee, 1987.[153]



2.6 *Histology Of The Normal And Aneurysmal Aorta*

An elastic artery such as the aorta is constructed from three characteristic layers (Figure 2.5a). From the luminal surface, a tightly packed monolayer of endothelial cells abuts against the internal elastic lamina to form the first layer, the tunica intima. This inner layer is in contact with the blood, its constituent cells and molecules, and the endothelial cells act as a conduit for biochemical interactions between the vessel and its contents.

The second layer, the tunica media, is bounded by the internal and external elastic laminae. This is the major structural layer of the artery, consisting of concentric rings known as elastic lamellae. Each lamellae is bounded by thick elastin fibres and contains circumferentially arranged collagen fibres, a network of fine elastin fibrils and a layer of smooth muscle cells amongst the structural components (Figure 2.5b).

These components give the vessel its ability to withstand and recoil against the pressure exerted by blood pulse waves.[154] Elastin gives the arterial wall the ability to distend and reform constantly, and its half-life of around seventy years ensures elasticity throughout the average lifespan. Collagen fibres act as the mechanical scaffolding, providing a strong framework within which maximal stretch may occur. Smooth muscle cells act principally to synthesise and maintain extracellular matrix integrity, and to a lesser extent than the intimal endothelial cells, are involved in modulating the biochemical status of the aorta.

The third and outermost layer is the tunica adventitia. This consists mainly of collagen fibres and fibroblasts. Also seen in this layer are small blood vessels termed vasa vasorum, which provide the outer regions of the artery with a blood supply.

Histologically, the aortic aneurysm is characterised by a number of marked changes in the arterial wall. The intimal layer, including the vast majority of endothelium, is absent. The medial layer is significantly thinned, and the content of this zone of the vessel wall is proportionately altered. The adventitial layer is significantly increased, contributing entirely to an overall thickening of the artery wall. Also notable is an increase in the number of adventitial vasa vasorum[155] (Figure 2.6).

Figure 2.5. **Histology of the normal aortic wall. (a) Intimal (I) , medial (M) and adventitial (A) layers. (b) High power micrograph of elastin van gieson stained medial layer, illustrating the elastic lamellae. Elastin is stained black, collagen pink and smooth muscle cells yellow.**

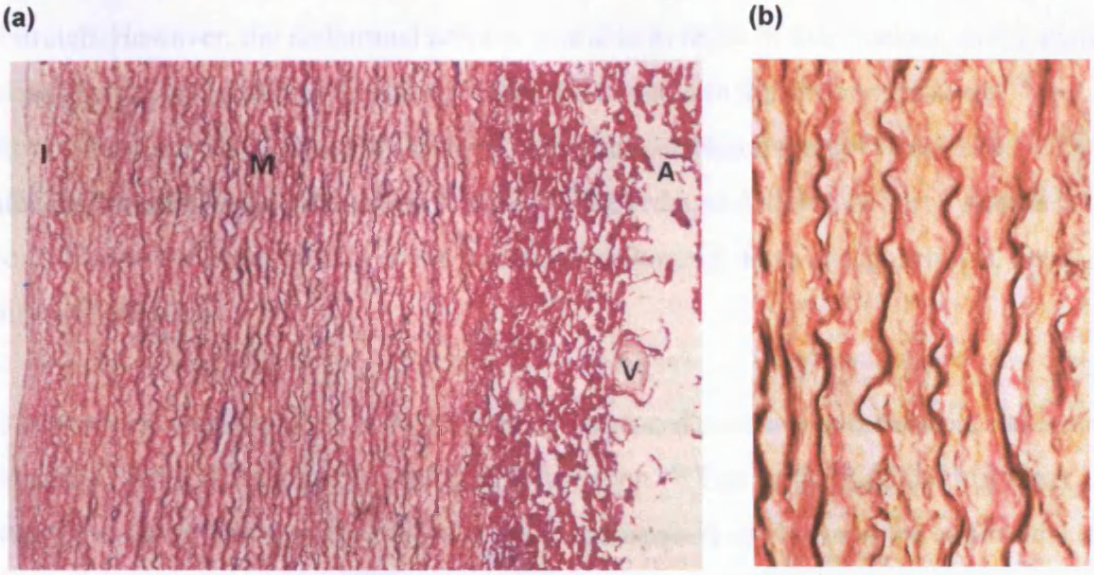
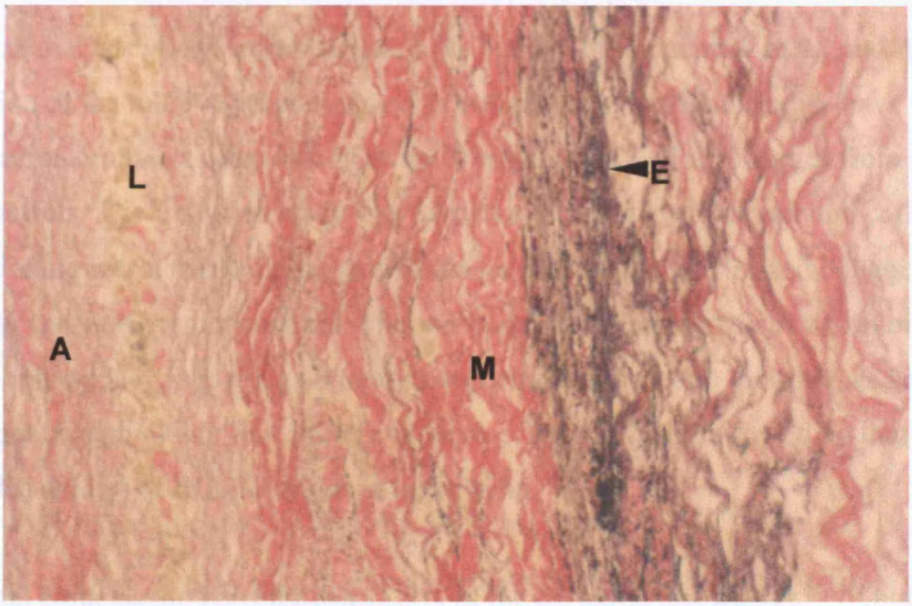


Figure 2.6. **Micrograph of elastin van gieson stained wall of abdominal aortic aneurysm, illustrating the histological changes characteristic of the disease. The intima is absent, the media (M) thinned with profound loss of elastin (E, arrowed) and the adventitia (A) thickened yet sparse and disorganised. Also visible is a patch of inflammatory leukocytes (L).**



2.7 *Influence Of Blood Flow*

As has been discussed, the aorta is subject to high tensile pressure, exerted from within by the pulsatile blood flow. The structural composition of the thoracic aorta equips it to withstand this pressure, with the medial elastic lamellae countering the distension by providing strength and stretch. However, the abdominal aorta is less able to resist in this manner, as the number of elastic lamellae reduces progressively from the thoracic to the abdominal aorta.[156] In addition, elastin fibres degenerate naturally, and unlike collagen are not remodelled. This age-related decrease in vessel distensibility, added to the reduced abdominal aortic stretch is likely to contribute to the susceptibility of the infrarenal abdominal aorta as a prominent site for aneurysm formation.

This anatomical predisposition is augmented by increased pressure in the abdominal aorta, generated by reflected waves from the iliac bifurcation.[157] The heightened and turbulent pressure generated here may contribute to the fragmentation of elastin in the abdominal aorta. Evidence to substantiate this theory is provided by a study on below-knee leg amputees, in which the asymmetric flow pattern produced by the abnormal vascular structure was associated with an increased incidence of aneurysms.[158] The effect of blood pressure on vessel expansion was confirmed in a study by Thompson *et al*,[159] who demonstrated that exclusion of an AAA from the circulation by endovascular repair reduced aneurysm diameter.

2.8 *Biochemical And Extracellular Matrix Changes*

Biochemical analysis allows examination of the content of the normal aorta, and the quantification of the relative proportions of its components. Detailed studies have been made of the extracellular matrix composition of aneurysmal aorta, and its changes from that of age-matched controls. The first such study was carried out in 1970 by Sumner and colleagues.[160] The main finding was a marked reduction in the percentages of elastin and collagen in AAA compared to non-aneurysmal controls. The study also described a redistribution of those structural components, indicating adventitial deposition and medial thinning, as demonstrated by histological examination. Since then a number of groups have followed up that work with more detailed studies of ECM changes in aneurysms.

In the normal aorta elastin constitutes 15-35% of the de-fatted dry weight,[161,162] but in the AAA this can be as low as 5-8%.[161-163] Expressed as a ratio of the total insoluble extracellular

material, the elastin concentration of the AAA has been reported to be reduced by 90%,[164] and in a similar study the proportion of insoluble elastin was found to have decreased from 12% in controls to 1% in AAA.[165]

These data suggested reduction in elastin concentration because of its destruction, most likely by proteolytic means. However it has been claimed by some that this is not necessarily the case. Minion and colleagues[166] reported that the apparent reduction was a result of the dilution of elastin within an increasing total protein content, due to increased synthesis of collagen and other matrix components, and not exclusively due to proteolysis. Significantly however, elastin gene expression has been analysed in AAA and has been reported to be unchanged.[115] Furthermore, urinary elastin-derived peptides, degradation products of elastin destruction, were elevated in AAA patients compared to controls,[167] strongly suggesting increased elastin proteolysis was occurring in aneurysmal aorta.

In contrast, the elastin-associated microfibrillar proteins such as fibrillin were shown to increase in concentration in AAA tissue by around 20%,[164-166] although the precise identification and role of these proteins has not yet been defined. Similar findings have been obtained for collagen, the principal structural fibre of the aortic wall. Collagen is arranged in a network, in the arteries consisting mainly of types I and III,[161] with type III being responsible for the majority of structural strength.[168] The loss of elastin fibres during aneurysm formation removes the main load-bearing components of the aorta[169] and places that burden on the collagen network, a phenomenon termed collagen recruitment.[170]

Analysis of aortic collagen revealed that the levels increase in aneurysm tissue compared to control aorta. The content has been reported to be between five[166] and three-fold greater[171] in AAA. In terms of dry weight, collagen content has been reported to increase from 62% in controls to 84% in AAA,[168] and in a similar study was described as having increased by 77% when expressed as a total volume fraction.[172]

The association of collagen deposition and aneurysmal expansion has led to speculation of a causal relationship, but it is more likely that collagen synthesis is a compensatory response to the loss of elastin integrity.[173] This theory is supported by data which demonstrated increased collagen mRNA synthesis and fibril deposition in the wall of unruptured aneurysms,[174] and

was further augmented by the fact that stretch is known to be a stimulus for the synthesis of ECM components by vascular SMCs.[175]

One of the most interesting findings regarding increased collagen synthesis in AAA was that the ECM changes seen were not confined only to the dilated, diseased part of the aorta, but were found in tissue proximal to the aneurysm site.[171] This observation supported data obtained in a separate study which reported increased mean diameters of the carotid, femoral, brachial and popliteal arteries in AAA patients.[129] Together, these studies provided powerful evidence that AAA may be a localised manifestation of a systemic predisposition to vascular dilatation.

The development of an aortic aneurysm brings about a substantial change in the amount and proportion of various ECM components. Contemporary evidence does not yet clarify whether these observed changes represent primary aetiological events, or are symptomatic of a broader picture of cellular interaction, proteolysis and inflammation.

2.9 *Influence Of Cell-Cell And Cell-Matrix Interactions*

Cell-matrix interactions, mediated by integrins, have not been studied in AAA tissue. Their role in vascular tissue is widespread[176] and disturbance in these interactions is seen in other vascular pathologies. One interesting aspect involves the critical nature of the integrin $\alpha_v\beta_3$ to endothelial cell invasion of tissue matrix in angiogenesis[177] The same integrin has been shown to directly bind the enzyme MMP-2[178] and play a role in localisation of the proteolytic activity to the surface of invasive cells.

The integrin $\alpha_3\beta_1$ has also been shown to modulate MMP-2 expression.[179] Blockage of this integrin with monoclonal antibodies led to increased MMP-2 production and invasiveness in tumour cells. Blockage of $\alpha_5\beta_1$ stimulated expression of MMP-1 and 3 in synovial fibroblasts.[55] A similar study showed that binding of collagen to integrin $\alpha_2\beta_1$ induced MMP-1 expression in osteogenic cells.[57]

Interaction with ECM components via integrins is known to regulate various aspects of cells such as adhesion, phenotypic change and migration. This has been demonstrated in SMCs,[180] where integrins of the β_1 family largely mediated their interactions with the ECM.[181] Other

cell-cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), have been demonstrated in atherosclerotic lesions[182,183] and are known to play fundamental roles in the interaction of leukocytes with the vascular endothelium.[184] Such involvement with ECM adhesion and degradation suggests that the integrins may be a worthwhile target for study in AAA disease.

2.10 *Proteolytic Influence*

The alterations in ECM components observed in aneurysm tissue suggested that radical remodelling and turnover of various components had occurred. Degradation of ECM *in vivo* is principally performed by the matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases which together may degrade all ECM components.[11] Refer to Chapter One.

2.10.1 *Elastolysis*

Bussutil *et al* were the first group to record increased proteolytic activity in AAA tissue.[151] Interest in the role of proteolytic enzymes has stemmed particularly from the apparent loss of elastin,[160] an event thought to occur early in aneurysm formation.[185] To this end, elastolytic enzymes were sought in AAA tissue. Brophy *et al* [186] described increased MMP-9 activity in tissue extracts, a known elastase[187,188] and the most prominent MMP detectable. This finding was confirmed by Reilly *et al*[189] who also reported that tissue extracts were capable of degrading intact elastin. Zymography studies of such extracts revealed the gelatinases (MMP-2 and 9) as the most common MMPs in AAA tissue, both known elastases.[187,188]

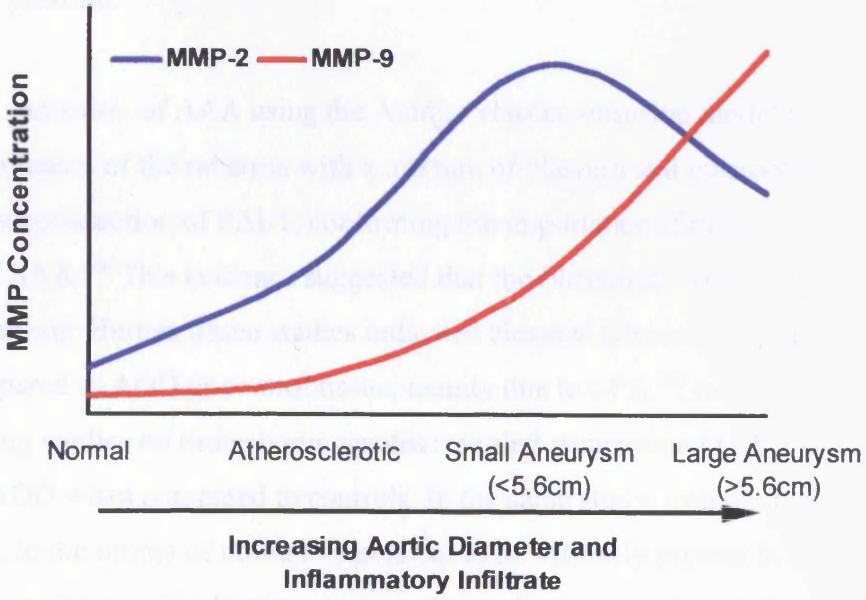
MMP-9 production has been histologically localised to infiltrating macrophages[190] seen particularly in the media and adventitia of AAAs. These cells were concentrated at sites of medial elastin damage, providing strong evidence for a causal relationship. Subsequent studies confirmed the presence of increased MMP-9 in AAA compared to controls [191-195] and further defined its source as infiltrating macrophages,[185,196] cementing its importance as a proteolytic influence in aneurysm formation.

The ubiquity of MMP-9 in AAA tissue has made it a prominent target for study by many groups and its co-localisation with fragmented elastin underscored these efforts. However, MMP-9 is not the only elastolytic enzyme present. MMP-2 has also been demonstrated in AAA tissue.[190] This is perhaps less of a surprise, as MMP-2 is expressed constitutively by

many cell types and has been found in both normal and AOD tissue at similar levels.[190] MMP-2 is produced by SMC's and other cell types, including endothelial cells and fibroblasts.[185,197]

A study fundamental to the approach of this thesis was carried out by Freestone *et al*,[185] which analysed the MMP content of AAA's of different diameters. In established AAA's, MMP-9 was the dominant elastase present, as has already been discussed. However, their important discovery was that in small aneurysms, MMP-2 was the most prominent enzyme and that this diminished in proportion as the aneurysm enlarged (Figure 2.7). The reduction in MMP-2 and increase in MMP-9 in AAA tissue appeared to correlate with the diameter of the aorta and the density of the inflammatory infiltrate.[198]

Figure 2.7 Graphical illustration of the proportion of MMP's in aneurysm tissue related to the diameter of the aorta and the density of the inflammatory infiltrate. Adapted from Freestone *et al*, 1995.[185]



The Freestone *et al* study suggested MMP-2 as the protease responsible for the early events of elastin degeneration, followed by an inflammatory cell-led phase of elastolysis and collagenolysis, mediated primarily by MMP-9 and other MMP's. However, production of MMP-2 is not sufficient for proteolysis. As was described in Chapter One, MMP's are secreted from cells as latent zymogens which must undergo activation.[11] MT1-MMP, the main physiological activator of MMP-2, is produced by vascular SMC's and macrophages[199] suggesting a likely role for this enzyme in the MMP-2-mediated elastolysis seen in AAA.

Other MMPs with elastase activity include MMP-8 and 12. The elastolytic activity of the latter was confirmed in a study in which MMP-12 deficient mice were shown to be incapable of efficiently degrading elastin.[200] MMP-8 is produced by macrophages located in potentially unstable regions of atherosclerotic plaques, but to date has not been reported in AAA tissue. MMP-12 has been shown to be produced by alveolar macrophages in lung disease such as emphysema[15,18,201] and has recently been associated with macrophages within the medial and adventitial layer of AAA tissue.[202]

Certain serine proteases have elastolytic activity and have been implicated in AAA pathogenesis. Perhaps the most widely studied is plasmin, an 80kDa protease which is part of the fibrinolytic system.[64] Plasmin is a powerful activator of some MMPs[52,62,66,67,114] and u-PA/u-PAR expression has been demonstrated on monocytes/macrophages,[203] suggesting a role in ECM turnover. Indeed, macrophage-mediated degradation of ECM has been shown to be dependent on plasmin.[204]

Experimental induction of AAA using the Anidjar elastase-infusion model in rats[205] was also achieved by infusion of the rat aorta with a mixture of plasmin and macrophages,[205] and blocked by overproduction of PAI-1, confirming the importance of the fibrinolytic system in this model of AAA.[206] This evidence suggested that the fibrinolytic system may play a role in AAA pathogenesis. Human tissue studies indicated elevated fibrinolytic activity in AAA tissue as compared to AOD or control tissue, mainly due to t-PA.[207] Interestingly, immunoblotting studies on tissue homogenates revealed an increase in t-PA in AAA but a reduction in AOD when compared to controls. In the same study, immunohistochemistry localised t-PA to the intima of normal vessels but to be diffusely present in intima and media of AAA tissue. u-PA was localised to mononuclear cells associated with the adventitial side of the diseased artery.[207]

Separate studies have confirmed the presence of elevated levels of t-PA in AAA tissue[208-212] and have suggested less widespread expression of PAI in AAA tissue, implying a tendency towards plasmin mediated proteolysis.[210] The co-localisation of fibrinolytic activity with MMP-9 production at sites of medial elastin destruction and neovascularisation[190,209,213] further supported a role for the former in aneurysmal ECM degradation.

2.10.2 *Collagenolysis*

The evidence which has led to the theory that elastolysis is an early event in AAA pathogenesis also suggests that collagenolysis is a later event, possibly secondary to the influx of inflammatory cells. As has already been explained, Bussutil *et al* were the first to demonstrate elevated levels of collagenase (MMP-1) in AAA tissue[151] and this has since been confirmed by others.[168,192,214,215] The production of MMP-1 in AAA is mainly confined to the adventitial layer[215] where it appears that the cellular source is predominantly mesenchymal, with a small amount being produced by vasa vasorum endothelial cells.

Other collagenolytic enzymes studied include MMP-3, which has been detected in extracts of AAA tissue.[193] MMP-3 is also found in supernatants from cultured SMCs derived from AAA vessel wall.[216] The role of MMP-3 is less obvious than that of MMP-1, but it is known to degrade a wide range of substrates, such as basement membrane proteins and proteoglycan core proteins.[11] MMP-3 is also known to activate other MMPs as part of an activation cascade, for example the superactivation of MMP-1.[60] See Section 1.4 for a more detailed description of these properties.

2.10.3 *Cellular Source Of Proteases*

The cells of the inflammatory infiltrate are the most prominent cells observed in the established aneurysm.[217,218] Macrophages in particular are thought to be a major source of matrix degrading enzymes; their capacity for MMP production has been well documented[219-221] and they have been identified as one of the main sources of elastolytic MMP-9 in aneurysm tissue.[190] Macrophages are known to be capable of secreting a range of proteolytic enzymes with the ability to degrade most ECM components.[222,223] A report by Shapiro *et al*[224] demonstrated that during differentiation from monocytes to macrophages upon migration into tissue, the secretion of serine proteases reduced and the controlled expression of MMP genes, particularly MMP-1, 3 and 9 was enabled. The same study reported that native and denatured collagen types I and III markedly increased macrophage MMP-1 secretion *in vitro*. This reaction occurred via a direct cell-matrix interaction, and may be considered relevant in AAA disease given the increased collagen turnover occurring in the adventitia.

It is likely that other macrophage-derived MMPs are important in aneurysm progression, such as MMP-7 and 14, although this is not yet supported in the literature. In the previous section, the environment of the degenerating aortic wall was described as being complex, with altered

levels of cytokines and breakdown products present, all of which may have contributed to the modulation of MMP expression by infiltrating cells.

Lymphocytes are known to be capable of MMP expression under certain circumstances,[225,226] but no published evidence has suggested this in AAA. However, their presence may indirectly drive matrix destruction via their cytokinetic influence on resident and infiltrating cells. Direct contact between T lymphocytes and macrophages has been shown to stimulate MMP-9 production in the latter,[227] although other T cell products, such as interferon-γ and IL-4, inhibit macrophage MMP production and stimulate TIMP-1 release.[227,228] Elevated IFNγ levels have been demonstrated in AAA tissue,[229] providing evidence that the lymphocytes may be attempting to reduce MMP production as a protective response.

Aortic endothelial cells are absent from the aneurysmal intima, but angiogenesis in the adventitia and media,[230] providing a new population of endothelial cells, may influence AAA pathogenesis, as proliferating and migrating endothelial cells produce MMPs.[192,231] It is important to note that the "missing" cells from the intima may have influenced initial proteolysis, but by their absence are impossible to study. Similarly, the newly arrived vasa cells may play a part in later stages of ECM breakdown. However, the association of inflammatory cells with vasa endothelium in AAA[192] suggests that the latter, like lymphocytes, might play a less direct role in ECM degradation, but through expression of adhesion molecules for example, may attract and direct the action of other cells.

One other main cell type exists in normal and aneurysmal aorta: the fibroblast. Its location in the outer adventitia has led to scant regard for its involvement in medial degeneration in AAA. However, tumour studies have shown the ability of the fibroblast to secrete MMPs,[232] and their production of MMP-2, in conjunction with MT1-MMP is thought to be crucial in tumour invasion.[233] No experimental data has been presented to support a role for adventitial fibroblasts in AAA to date.

The level of production of MMPs is generally very tightly controlled at the level of gene expression.[51] This is readily influenced by inflammatory cytokines, for example the increase in expression of MMP-9 in response to IL-1β or TNFα[33] and the down-regulation in response to TGF-β or IFNγ.[227,228] The role of MMPs in cytokine signalling is becoming more complex,

with their involvement as processors of TNFα[234] and the inhibition of TNFα secretion by TIMPs[235] recent examples. A new family of enzymes, termed sheddases or adamalysins, is also thought to consist of MMPs, and are involved in the cleavage of cell surface receptors, with or without bound ligand, as a means of signalling control.[236-238]

Elevated expression of MMPs need not mean increased proteolysis. As was described in Chapter One, the action of the enzymes depends on further regulatory mechanisms, such as activation and inhibition. The tissue inhibitors of metalloproteinases (TIMPs) provide physiological control of MMP activity and it was speculated that the level of TIMP may be reduced in AAA.[239] This has been shown to be untrue[240] and in fact, many groups have reported increased TIMP levels,[192,196] probably due to their co-expression with MMPs.[219,221,232]

2.11 *Inflammatory Infiltrate*

The changes in ECM components and the increase in proteolytic activity observed in the wall of AAAs is accompanied by a dense infiltrate of cells characteristic of an inflammatory response.[185,218,241,242] The principal contents of this infiltrate are B and T lymphocytes and macrophages. Although it is unclear whether the presence of these cells is causal in AAA disease, it can be said with some certainty that they have the capacity to influence vessel wall homeostasis in a number of ways.

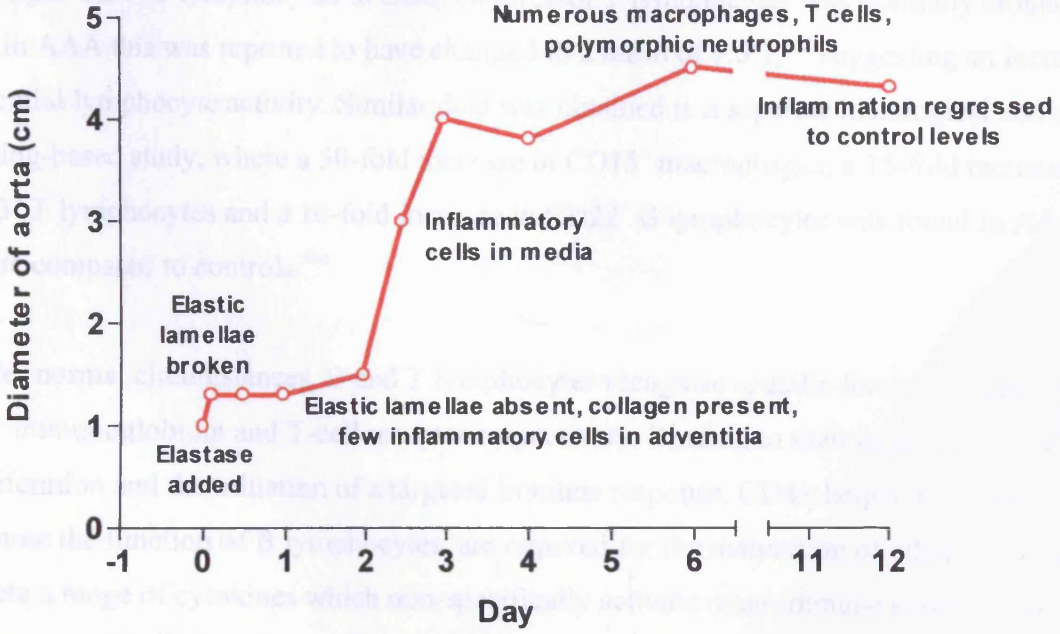
As was described in the previous section, macrophages and lymphocytes are able to secrete ECM degrading enzymes such as serine proteases and MMPs, and hence cause structural damage.[222,223] In addition, their function as mediators of immune responses and their production of cytokines may contribute to undesirable localised cellular responses both by paracrine action on resident aortic mesenchymal cells or by autocrine mechanisms amongst themselves, an aspect well described in atherosclerosis.[243] Histological analysis has revealed that the majority of the infiltrate resides in the medial and adventitial layers,[244-246] but is usually most pronounced in the adventitia.[217] The extent of loss of SMCs and elastin fibre fragmentation has been shown to correlate strongly with the degree of infiltration,[247,248] as has the number of vasa vasorum.[155]

2.11.1 *Inflammatory Aneurysms And The Importance Of Leukocytes*

A proportion of between 3 and 10% of AAAs are referred to as inflammatory aneurysms, as the infiltrate is particularly dense and is accompanied by extreme wall thickening and adherence of the aorta to surrounding abdominal organs. This phenomenon was first described by Walker *et al* in 1972[244] but it remains unclear whether this represents a distinct aetiology or a higher level of severity. Age of onset is frequently earlier[249] and evidence suggests an association with a particular HLA-DR polymorphism[250] resulting in heightened sensitivity to disease-related antigen. A recent study in Japan has identified the antigen HLA-DR2(15) as a significant risk factor for AAA.[251]

The potential importance of the infiltration of leukocytes in the induction and progression of aneurysmal disease was illustrated in a series of experiments using the Anidjar model of AAA. The induction of an aneurysm in rats by a single two hour local infusion of elastase into the aorta[205] was accompanied by two distinct phases of inflammatory cell influx.[252] Initial elastase-mediated fragmentation of elastin was associated with an initial moderate increase in aortic diameter (Figure 2.8), then a 2-3 day period of stability. This was followed by a second dramatic enlargement, during which a 300% increase in diameter occurred, and a second apparent stabilisation. This second phase was associated with the presence of inflammatory cells in the aortic media, particularly macrophages and lymphocytes, plus occasional neutrophils. The latter stabilisation phase correlated with regression of the infiltrate.[252]

Figure 2.8 Aneurysmal expansion in rat aorta following infusion with elastase, illustrating the phases of ECM destruction and inflammatory cell infiltration. Adapted from Dobrin *et al*, 1996.[252]



Such expansion was inhibited by treatment of the rat aorta with the immunosuppressive agents cyclosporine or methylprednisolone,[252] and reduced in rats treated with an antibody to CD18, the leukocyte adhesion molecule integrin β_2.[253] In both drug and antibody treated animals, a marked reduction in inflammatory infiltrate and elastin fragmentation was seen.

Non-specific activation of the immune system in the same animal model system by infusion of plasmin and thioglycolate resulted in a single phase, gradual enlargement in aortic diameter. Again, following comprehensive elastin fragmentation, regression of the infiltrate occurred. Furthermore, the infiltrate consisted only of macrophages, which were associated histologically with fractured elastic lamellae.[252] Although these experiments do not define whether the infiltrate is initiated by the foreign proteins elastase and plasmin, by fragmented elastin, or by some other stimulus, they do serve to illustrate the potential for inflammatory cells to influence aneurysmal expansion and disease progression.

Immunohistochemical phenotyping analysis of the infiltrating cells in humans has revealed that inflammatory cells were almost completely absent in the wall of normal, control aortas.[254]

In AAA tissue the most prominent inflammatory cell was the $CD3^+$ T lymphocyte, where 66% of these were found in the adventitia.[217] $CD19^+$ polyclonal B lymphocytes constituted around 25% of the total infiltrate in AAA,[217] and were also mainly located in the adventitia. The ratio of helper $CD4^+$ T lymphocytes to $CD8^+$ suppressor T lymphocytes was normally around 2:1,[69] but in AAA this was reported to have changed to a mean of 7.6:1,[217] suggesting an increase in potential lymphocyte activity. Similar data was obtained in a separate histological and cell sorting-based study, where a 50-fold increase in $CD15^+$ macrophages, a 15-fold increase in $CD3^+$ T lymphocytes and a 10-fold increase in $CD22^+$ B lymphocytes was found in AAA tissue compared to controls.[194]

Under normal circumstances, B and T lymphocytes recognise specific foreign antigens via their immunoglobulin and T-cell receptor respectively. Binding to their target causes cell proliferation and the initiation of a targeted immune response. CD4+ helper T lymphocytes promote the function of B lymphocytes, are required for the maturation of other T cells and secrete a range of cytokines which non-specifically activate other immune system cells such as macrophages.[255] T cell-mediated activation occurs via their expression of the CD40 ligand, a cell surface molecule which binds to the CD40 receptor borne by macrophages, B cells and dendritic cells.[256] Such an interaction is known to induce MMP-9 expression in macrophages[257,258] and antibody blockade of this signal has been shown to diminish atherosclerotic plaques in genetically predisposed mice.[259]

T lymphocytes tend to be distributed throughout the circulatory system, constantly in motion until contact is made with antigen. In contrast, B lymphocytes aggregate in lymphoid tissues and nodes where processed antigen is presented to them by other cells such as macrophages and dendritic cells. It is unclear whether the lymphocytes observed in AAA tissue provoke the initial immune response or whether a non-specific stimulus, such as the phagocytosis of elastin fragments triggers the lymphocyte influx. In common with histological analysis of other immune responses, features of non-specific and specific cellular activity are seen in AAA.

In a study by Brophy *et al,*[218] elevated levels of immunoglobulins were detected in AAA tissue compared to controls. This suggested antibody production by activated B lymphocytes in response to a specific antigen. However, a separate study reported the absence of immunoglobulin in both control and AAA tissue,[260] and predicted that a specific immune

response plays no role in the pathogenesis of AAA. Confusingly, a recent study to determine the usage of immunoglobulin heavy chain genes, and hence the specificity of antibody production in AAA derived B lymphocytes revealed no cell clonality and unrestricted gene usage.[261] This appeared to partly support the conclusions of both of the above studies, leading to the hypothesis that whilst immunoglobulin production does occur, a specific, antibody-driven immune response is not present in AAA disease.

The macrophages observed in AAA tissue are found throughout the vessel wall, often associated with lymphoid aggregates, where it is possible that they are presenting processed antigen. The role of macrophages in immune responses is varied, acting as non-specific phagocytic cells for the removal of non-self bodies and for the presentation of antigen to $CD4^+$ helper T lymphocytes and B lymphocytes to illicit a targeted immunological response.[255] It is unclear whether a non-specific stimulus is responsible for the macrophage infiltrate in AAA or whether they are chemotactically recruited by CD4+ T lymphocyte signalling. The breakdown product of elastin fragmentation, elastin derived peptides (EDPs), have been shown to be chemotactic for cells of the monocyte lineage *in vitro*.[262-264]

2.11.2 *Production Of Cytokines By Inflammatory Cells*

Macrophages may be responsible for indirect modulation of proteolysis via their paracrine actions on aortic mesenchymal cells. Vascular smooth muscle cells are capable of secreting a panel of MMPs following cytokinetic stimulation,[33] particularly by IL-1β and TNFα. These cytokines, associated with inflammation, have been demonstrated at elevated levels in homogenates of AAA wall as compared to control tissue,[265] as have IL-6,[229] interferon γ,[229] monocyte chemoattractant protein-1 (MCP-1)[266] and IL-8.[266] Macrophages are the main source of IL-1β *in vivo*[267] although B lymphocytes, endothelial cells and fibroblasts may also secrete this product when stimulated by IL-1β, an interesting phenomenon which may initiate a positive feedback loop involving the vascular cells, serving to amplify local inflammation.[268] IL-1β has been shown to be a mitogen for vascular SMCs[269] and may increase their production of collagen.[270]

TNFα is produced primarily by monocytes/macrophages,[267] although it is also produced by lymphocytes and vascular SMCs,[271,272] again capable of initiating a positive feedback reaction in the latter.[272] TNFα has been shown to be capable of stimulating MMP expression in

vascular SMCs[33] and has angiogenic properties, serving to stimulate endothelial cell proliferation.[273] The observation of macrophages accumulating in adventitial vasa vasorum of AAA tissue was perhaps further evidence of this effect, particularly in the context of increasing numbers of new adventitial vessels.[155,274] Such adventitial angiogenesis may have served to directly increase the inflammatory infiltrate seen in the developing aneurysm by providing direct circulatory access to the diseased tissue.

Macrophages are also the main source of IL-6 *in vitro*,[275] along with fibroblasts, endothelial cells and SMCs. IL-6 acts primarily to activate B and T lymphocytes during an inflammatory response.[229] Vascular SMCs produce large quantities of IL-6 in the presence of IL-1β, further activating lymphocytes and enhancing immunoglobulin production by B cells,[275] perhaps helping to explain the increased immunoglobulin found in AAA tissue as mentioned earlier.[218,261] Similarly, interferon γ also activates lymphocytes and induced MHC class II expression on endothelial cells and vascular SMCs, thus enhancing antigen presentation.

Other cytokines elevated in AAA disease include IL-8 and MCP-1.[82] IL-8 is produced mainly by monocytes/macrophages and is highly chemotactic to neutrophils, lymphocytes and endothelial cells.[276] It has potent angiogenic properties,[277] and is a mitogen and chemoattractant for vascular SMCs.[278] MCP-1 is also produced by tissue macrophages[279] and has specific chemoattractant and activating properties for monocytes.[280] MCP-1 is fundamental to the recruitment of macrophages in atherosclerosis, as demonstrated by reduced lesion size in MCP-1 receptor-deficient mice fed an atherogenic diet.[281] Both of these cytokines were produced in increased quantities from tissue explants of aneurysmal aorta compared to normal controls[266] and were both produced predominantly by macrophages on histological examination. Furthermore, *in vitro* studies have demonstrated that endothelial cells and vascular SMCs stimulated with IL-1β or TNFα may secrete IL-8.[282,283]

Whilst the infiltrating cells are the most obvious manifestation of an immunological response in AAA disease, it is important to note that other, more subtle changes are occurring in the tissue. One of the prominent mediators of the inflammatory response is the mast cell, a static, highly granular cell which stores large amounts of biologically active compounds.[284] Degranulation of mast cells occurs when immunoglobulin E (IgE) in conjunction with antigen binds to receptors on the cell surface. IgE is produced as a defence against non-infectious

antigens by B lymphocytes.[255] The contents of mast cell granules have many functions, but serve generally to improve circulatory access to the tissue and to attract other immune system cells.

Vasodilators such as histamine and nitric oxide are released during mast cell degranulation[284] along with cytokines such as IL-1β and TNFα to increase inflammation and stimulate influx of phagocytic cells as already described. Further products include serine proteases such as plasmin, a point of some importance with relevance to aneurysm aetiology, as mast cell products have been shown to participate in elastolysis[285] and to activate MMP-1 and 3.[286]

A study by Bakos *et al*[287] clearly showed a four-fold increase in the number of mast cells per unit area in AAA tissue as compared to controls. Interestingly, the aneurysm mast cells were degranulated to varying degrees, but frequently this was partial. Whether this represented selective release of certain components or a mild stimulus causing a mild inflammatory response was unclear. However, for degranulation to occur at all, IgE-antigen binding would appear to be necessary.

2.11.3 *Autoimmunity And Infection As Causes Of AAA*

A theory that has recently taken shape, states that AAA is an autoimmune condition, resulting from an inappropriate response to aorta-specific autoantigens.[288] IgG reactive to 80kDa and 40kDa aortic matrix components have been isolated and sequenced by Tilson and colleagues,[289,290] and these authors have proposed that this aortic aneurysm antigenic protein-40 (AAAP-40) was an elastin-associated microfibrillar component, with homology to other microfibril-associated glycoproteins.[288] They also speculated that the putative calcium binding properties of such a protein, if affected by auto-antibody binding *in vivo*, may result in disrupted microfibril assembly. This situation may be induced by EDTA *in vitro*,[291] and such an approach awaits further research.

Infectious agents have long been proposed to play a role in atherosclerosis and other vascular pathologies, where the initiation of tissue damage is thought to provoke an immune response consistent with plaque formation.[292-298] Such speculation has been applied to AAA, where cytomegalovirus[299] and *E.coli*[300] have been reported in the wall of the diseased vessel, associated with a strong inflammatory response. One recent adaptation of this theory involves

molecular mimicry, where homology between matrix proteins and pathogenic organisms results in immune response against the self proteins.[301,302] The AAAP-40 protein described earlier has homology to herpes simplex and *Trepanema pallidum*.[303]

Bacterial proteinases such as thermolysin have been shown to activate MMP-1, 8 and 9[304] by limited cleavage of their pro-peptide, but this has not been shown to have significance to any human pathology. The notion of infection causing AAA or other vascular diseases is unproven and remains an interesting topic for study. However, the lack of consistently detectable infection in AAA suggests that this approach may identify only a small subset of cases.

2.12 *Atherosclerosis*

A common hypothesis states that AAA is a product of severe atherosclerosis.[130] This suggestion is based on a number of pieces of circumstantial evidence, but is centred on the observation that aneurysmal degeneration is almost invariably accompanied by atherosclerotic lesions. The two pathologies share risk factors such as smoking, hypertension and hyperlipidaemia,[130,305] and commonly occur in the infra-renal aorta. They are both associated with a macrophage-rich inflammatory infiltrate, and both lesions contain enhanced levels of ECM degrading proteases, particularly MMPs.

If the two conditions are examined in more detail, other similarities emerge. Early proliferation of SMCs in AOD, which accompanies intimal damage,[306-308] is later followed by SMC loss and substantial medial thinning in the zone immediately beneath the plaque.[309] This SMC and elastin loss may represent early degenerative changes which become more profound to bring about aneurysmal dilatation. Interestingly, the SMC loss in AOD is reported to be associated with intimal apoptosis[310,311] in contrast to AAA where evidence suggests that apoptosis occurs principally in the media,[312] possibly contributing to the thinning of this layer.

However, from this point the two conditions appear to diverge. Progression of AOD occurs as lipid accumulation in the plaque increases, leading to fibrosis of the intima and occlusion of the vessel lumen.[313,314] Macrophages associated with the lesion become foam cells as they take up low density lipoprotein (LDL) via their scavenger receptors[315,316] to become a significant fraction of the lipid-rich core of the developing plaque. Severe fibrosis may cause calcification

and this further reduces blood and oxygen supply to the vessel wall, leading to necrosis and ulceration. This is associated with an increase in MMP activity, particularly at the extremities of the plaque, where degradation of the stable tissue may lead to thrombus formation and severe occlusion.[317]

These events are in marked contrast to AAA disease, where plaque formation is a relatively minor aspect compared to the massive degenerative events which permit dilatation. In an attempt to establish an aetiological link between AAA and AOD, Zarins *et al* induced aneurysms in monkeys by feeding a lipid-rich, atherogenic diet for 12 months,[131] after which they were transferred to a normal diet. 5 of 443 animals developed an AAA, in whom the authors speculated that regression of the plaque removed structural support for the thinned media, with resultant aneurysmal dilatation. In a separate study, aneurysms were induced in the aortas of rats by localised infusion of elastase, after the method of Anidjar *et al*.[253] The study compared the response in rats genetically predisposed to hypertension with normotensive animals. Aneurysmal expansion of the aorta in the hypertensive rats was not substantially greater than that in the normotensive rats. These two studies suggested that whilst atherosclerotic changes may have affected the expansion rate of an aneurysm, there was no evidence to suggest that the aetiologies of the two conditions were directly linked.

The emergence of the opinion that AOD and AAA are distinct aetiopathological entities occurred when researchers questioned how atherosclerotic changes could lead to occlusion in some patients and dilatation in others.[318] Primary among their concerns were the observations that the patient profile was different for AOD, with more women sufferers and a lower age of onset[132,318] and that AAA cases clustered in families.[106] Glagov *et al*[319] have suggested that AOD is secondary to AAA. They proposed that atherosclerotic changes occurred as a result of increased vessel wall exposure to blood borne atherogenic factors, as may occur in AAA where turbulence is high and shear stress is low.

Ward's observation of systemic arterial dilatation in AAA patients[129] is perhaps the strongest evidence of a distinct aetiological pathway for AAA. The peripheral arteries measured in this study are very rarely associated with atherosclerotic changes, suggesting that the dilatation is unlinked to AOD. Recent evidence has furthered neither hypothesis to the detriment of the other. Indeed, it is equally possible that both theories may be partially correct, with a proportion of AAA a secondary phenomenon to existing AOD, and the remainder a distinct

pathological entity. Much further research is required to delineate the aetiologies of the two disease processes.

2.13 *Rationale Of Thesis*

Previous investigations have defined AAA as a complex disorder with a multi-factorial aetiology. Familial clustering of the disease has implied a genetic predisposition, and the association of AAA with risk factors such as smoking suggested that an environmental contribution may be required to unmask the disease potential. A scheme of events has been derived from histological and biochemical studies, suggesting medial elastin damage to be an early event, followed by simultaneous collagen remodelling, inflammation and vessel dilatation. However, the use of end stage disease tissue in the majority of these studies has prevented the separation of primary aetiological events from secondary degenerative changes.

As described in Section 2.6, the cellular content of AAA tissue is substantially altered from the normal aorta. This has presented difficulty when attempting to assess the role of individual cell types in the disease process, as some are absent and some have been newly introduced. The cell which is present in the medial layer both before and after aneurysmal degeneration is the SMC. Vascular SMCs are known to be capable of producing MMP-1, 2, 3 and 9.[33,216,320-324] Increased production of MMP-2 has been demonstrated in SMCs derived from neointimal hyperplasia,[324] highlighting the capacity for these cells to become an integral part of pathological states. MMP-2 has been localised to SMCs in immunohistological studies of AAA tissue[185,197] and as already described, has been assigned as the dominant MMP in small aneurysms,[185] suggesting that the SMC may have a fundamental role in the initiation of ECM degeneration in AAA. Their capacity for MMP production, coupled with their location within the elastic lamellae, provides ample scope for elastin fragmentation, and the initiation of a proteolytic/inflammatory cascade.

In Section 1.4.3 the MT1-MMP-mediated activation and cell surface binding of MMP-2 was discussed in detail. Although this facet of MMP action has not yet been addressed in AAA, it is possible that membrane-associated activation of MMPs has a significant role in medial elastolysis and that the localisation of proteolytic activity on the surface of cells such as SMCs imparts a major influence on the initiation of AAA. The dynamic relationship between MMP-

2/MT1-MMP/TIMP-2, and their collective elastolytic capacity, formed the basis of the approach of this thesis, and the role of this enzyme system was considered.

From the preceding text, it is clear that the AAA represents an environment of enormous complexity, with a range of cell types contributing to various MMP production. The relatively clear picture of the identity and source of MMP production has provided little information on the initiation and regulation of this cascade, nor of the cellular interactions taking place. Furthermore, the controlled MMP expression associated with SMC migration in atherosclerotic disease has clouded the issue still further, contrasting strongly with the seemingly uncontrolled ECM destruction seen in AAA.

The aim of this thesis was to investigate an initiating factor of aneurysm formation. Thus, the present work aimed to examine the MMP expression of isolated SMCs derived from aneurysm tissue and compare them to controls. By separating the cells from their cytokinetic and cell contact-mediated signals *in vivo*, it was hoped that any primary differences which may contribute to the pathogenesis of AAA would be revealed.

Initial studies aimed to identify the MMPs being produced in the aneurysmal and control aortic tissue by immunohistochemical and *in situ* hybridisation techniques. Particularly of interest were the activators and inhibitors of elastolytic MMPs, whose presence or absence may be important indicators of ECM degeneration.

It was proposed that any phenotypic differences detected in vascular SMCs should be reflected both in other mesenchymally-derived cells and in other vascular tissue taken from AAA patients. To answer this question, and to determine whether AAA is a localised manifestation of a systemic predisposition, fibroblast cultures were derived from skin samples taken from AAA patients and their MMP production compared to controls as described above. Similarly, samples of inferior mesenteric vein were used to assess MMP production in other vascular tissue.

Elastin is known to fragment naturally over a number of years *in vivo*, leading to the reduced elasticity of blood vessels and skin. The breakdown products, elastin derived peptides (EDPs) are known chemoattractants for inflammatory cells and may play a role in provoking the AAA-associated inflammatory response. Preliminary work has suggested that EDPs may also

influence MMP production and activation. This was examined by assessing the influence of EDPs on cell-derived MMPs and purified enzyme.

Finally, using a modification of a porcine aortic *in vitro* model of aneurysm formation, the theory that MMP-2 is critical in aneurysm initiation was tested. In the original model, pancreatic elastase was used to initiate medial degeneration.[325] However, in the present work, elastase was replaced by MMP-2. It was hoped that these experiments further defined the role of medial SMCs and elastolysis in the initiation of aneurysmal disease.

CHAPTER THREE

Materials and Methods

Unless expressly stated, all reagents included in these methods were purchased from Sigma, Poole, UK.

3.1 *Tissue Culture*

Tissue biopsies were obtained from patients undergoing AAA repair or aorto-bifemoral grafting for occlusive disease. Non-atherosclerotic tissue was obtained from organ-donor cadavers. All samples were taken 2cm distal to the left renal vein for consistency. Abdominal skin samples and inferior mesenteric vein samples were taken from the same patients where possible. Saphenous vein was obtained either from cardiac bypass patients or carotid endarterectomy patients at the time of operation. Approval for the collection of such samples was granted by the Ethical Committee of the Leicestershire Health Authority.

3.1.1 *Smooth Muscle Cell Culture*

Under sterile conditions, the medial layer of the aortic biopsy was dissected out carefully and explants prepared. The explants were added to tissue culture flasks (Nunclon) with sterile smooth muscle cell medium (RPMI 1640 containing 50000IU/L penicillin/streptomycin and 2mM L-glutamine (all Gibco BRL, Grand Island, NY, USA) and 10% foetal bovine serum and maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C. The medium was partially replenished on alternate days, and the outgrowth of cells monitored. Cultures were passaged when approximately 80% confluent, by treatment with trypsin/EDTA until removed from the flask, and replated at a density of approximately 3500 cells per cm^2 into new flasks. Saphenous vein SMCs were prepared and maintained in an identical fashion.

3.1.2 *Fibroblast Culture*

Under sterile conditions, the epidermis and dermis were dissected carefully from the sub-dermal fat and connective tissue, and explants prepared. The explants were added to tissue culture flasks (Nunclon) with sterile fibroblast culture medium (DMEM containing 2mM L-glutamine, 50000IU/L penicillin/streptomycin, 2.5μM hydrocortisone, 100μM 2-mercaptoethanol (all Gibco BRL, Grand Island, NY, USA) and 10% foetal bovine serum and maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C. The medium was partially replenished on alternate days, and the outgrowth of cells monitored. Cultures were passaged when approximately 80% confluent, by treatment with trypsin/EDTA until removed from the flask, and replated at a density of approximately 3500 cells per cm^2 into new flasks.

3.1.3 *Porcine Aorta Organ Culture*

Porcine aortic tissue was previously used in the establishment of an *in vitro* model of aneurysm disease.[325] Segments of aorta were treated with elastase for 24 hours, followed by culture for 14 days. The elastase treated aortas developed changes characteristic of aneurysmal disease, in particular the degeneration of elastin fibres, and exhibited alteration in their protease production when homogenates were analysed by zymography. The present work used this model to investigate the ability of MMP-2 to initiate aneurysmal degeneration. The use of intact tissue permitted observation of the effects of the protease in an environment which maintained cell-cell and cell-matrix interactions, and more accurately reflected that found *in vivo*.

Porcine aortas were obtained from freshly slaughtered pigs and transported to the laboratory in sterile MEM containing 50000IU/L penicillin/streptomycin. Under sterile conditions fat and loose adventitial tissue was removed. Segments of aorta 1cm^2 were excised and pinned, intima uppermost, onto a polyester gauze support upon Sylgard resin (Dow Corning, Seneffe, Belgium) in a 6cm glass petri dish with lid. Each sample was denuded of endothelium by abrading the intimal surface with a sterile cotton bud. This prevented the formation of an endothelial-dependent neointima, as described by Koo and Gotleib,[326] thus eliminating SMC proliferation and migration which may influence medial homeostasis.

The tissue segments were divided into groups according to their treatment regimen. Selected cultures were fixed immediately in 10% formalin to preserve the untreated tissue architecture. Other cultures were pre-treated with 10000U porcine pancreatic elastase (Calbiochem, Nottingham, UK) or 1μg purified MMP-2 (Oncogene Science, Paris, France) for 24 hours, according to the method of Wills *et al*,[325] which was then removed by washing prior to culture. All were cultured in smooth muscle cell medium as described above, in a humidified atmosphere of 95% air/5% CO_2 at 37°C. The medium was replaced after 24 hours and every 48 hours thereafter, up to a maximum of 14 days. After the culture period, tissue samples were divided, one half snap frozen for homogenisation and one half fixed in 10% formalin for histological analysis.

3.2 *Immunofluorescent Staining Of Cultured Cells*

It was necessary to confirm the identity of the cells grown in culture. This was achieved by an indirect immunofluorescent staining technique to allow visualisation of unique cellular antigens. Murine primary monoclonal antibodies were purchased from Sigma (smooth muscle α-actin and macrophage) and Dako, Ely, UK (fibroblast). Fluoroscein isothiocyanate (FITC)-conjugated rabbit anti-mouse secondary antibody was purchased from Sigma.

Trypsinised, cultured cells were plated onto sterile, glass 18mm square cover slips, where they were allowed to adhere for 24 hours and then fixed by submersion in 4% paraformaldehyde (PFA) for 15 mins at room temperature. Residual PFA was removed by three washes in PBS. Cell membranes were disrupted by submersion in 50mM NH_4Cl for 10 mins then PBS containing 0.1% Triton X-100 for 15 mins. Cells were washed three times in PBS-T/5% BSA (PBS containing 0.1% Tween 20 and 5% BSA), with the last wash left on for 15 mins to block non-specific antibody binding.

Cellular identity was confirmed using the appropriate murine primary monoclonal antibody, diluted in PBS-T. 100μl of the primary antibody (1 in 400) was applied to each cover slip, which were incubated in a humid chamber for 1 hour. Three washes in PBS-T/5%BSA were performed to remove unbound primary antibody, and block non-specific secondary antibody binding. 100μl of an FITC-conjugated rabbit anti-mouse secondary antibody (1 in 100 in PBS-T) was applied to the cover slips, which were returned to a darkened, humid chamber for a further hour at room temperature. The cover slips were then washed three timed in PBS-T, twice in PBS and once in distilled water, prior to being mounted onto microscope slides using DABCO mountant[327] (90% glycerol; 10% PBS; 220mM 1,4-diazobicyclo-(2,2,2)-octane), and sealed with nail varnish.

Regions of immunological reactivity were visualised as a green fluorescence by mercury vapour lamp illumination on an Olympus BH-2 microscope and recorded using an Olympus OM-10 still camera (Olympus, Tokyo, Japan) and 64T slide film (Kodak, Rochester, NY, USA).

This protocol was modified for the identification of fibroblasts and macrophages, by using murine anti-human fibroblast primary antibody or murine anti-human macrophage antibody as

appropriate. Negative controls were included, using either no primary antibody or mouse IgG as a non-specific control.

3.3 *Zymography*

This technique is an extension of the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) methodology, using a discontinuous buffer system as originally described by Laemlli.[328] Visualisation of protease activity is permitted by the appearance of negatively stained bands of lysis on a gel containing a positively stained substrate, such as gelatin or casein.[329,330]

10% gels were prepared according to the recipes summarised in Table 3.1, from 30%/0.8% acrylamide/bis-acrylamide (Protogel, National Diagnostics, Atlanta, Georgia, USA); TEMED; collagen type IV denatured at 65°C for 1 hour; resolving buffer (1.5M tris, pH 8.8); stacking buffer (0.5M tris, pH 6.8); 10% SDS; 10% ammonium persulphate (prepared freshly); and distilled water. The electrophoresis tank was filled with tank buffer (25mM tris; 200mM glycine; 3.5mM SDS; pH 8.3). Samples were mixed with an equal volume of non-reducing loading buffer (62.5mM tris, pH6.8; 1.4M SDS; 45% glycerol; 0.025% bromophenol blue) prior to loading onto the gel and current application. All gels were prepared and run in the Mini Protean II electrophoresis system (Bio-Rad, Hemel Hempstead, Herts, UK) at a current of 30mA for approximately 4 hours.

Following electrophoresis, the gel was washed three times for 15 mins in 2.5% Triton X-100 to remove the SDS from the gel and allow the fractionated proteins contained within it to renature. The gel was then incubated at 37°C for 18 hours in zymogram buffer (50mM tris; 10mM calcium chloride; 50mM sodium chloride; 0.05% Brij 35S) to permit substrate proteolysis by the enzymes present. The gel was stained for three to five hours in Coomassie blue stain (25% methanol; 10% glacial acetic acid; 0.02% Coomassie blue R-250), an irreversible stain for protein. Enzyme activity showed up as clear bands against a dark blue background.

Inhibition of protease activity was used to confirm the type of proteases detected on the zymogram gels. 10mM 1, 10, phenanthroline was used as a specific MMP inhibiting compound due to its ability to chelate zinc atoms. Alternatively, 1mM PMSF was included as

a specific inhibitor of serine proteases, which may appear as lysis on gelatin zymograms or be involved in activation of Pro-MMPs.

Table 3.1 Contents of zymogram electrophoresis gels.

Reagent	Resolving Gel	Stacking Gel
dH_2O	3ml	6.1ml
Substrate (1mg/ml)	1ml	-
30%/0.8% Acrylamide/Bis	3.34ml	1.3ml
Resolving buffer	2.5ml	-
Stacking buffer	-	2.5ml
SDS (10%)	100µl	100µl
Ammonium persulphate (10%)	50µl	50µl
TEMED	5µl	10µl

3.4 *Enzyme Linked Immunosorbent Assay (ELISA) Tests*

Quantitative ELISA assays were performed to determine the concentrations of various MMPs/TIMPs in the media samples collected from cell lines and tissue homogenates. Commercially available ELISA test kits were purchased from Amersham (Little Chalfont, UK), and were used according to the manufacturers protocol.

The ELISA kits consisted of a 96-well plate, pre-coated with specific anti-MMP/TIMP antibody. Samples were applied to the wells and allowed to bind, along with samples of known concentration. Excess samples was washed from the wells, and an anti-MMP/TIMP antibody-horse radish peroxidase conjugate applied to the plate and allowed to bind. Excess antibody was removed by washing and TMB (3,2',5,5'-tetramethylbenzidine) added. This produced a colour change which was proportional to the amount of bound peroxidase. The reaction was stopped by the addition of sulphuric acid, and the extent of oxidation read spectrophotometrically at 450nm. The concentration of MMP/TIMP in the samples was calculated by comparison with a standard curve constructed from the samples of known concentration.

3.5 *Immunohistochemical Staining Of Tissue Sections*

Immunohistochemical staining of aortic tissue sections was performed to establish the location and level of MMP/TIMP production within the arterial wall. Both frozen and paraffin wax embedded tissue was used. The former has the advantage of better preserving antigen integrity, but suffers from some distortion of tissue architecture, particularly in calcified tissue. Wax embedded tissue yields more intact sections, but signal is commonly low, often demanding the use of antigen retrieval methods, such as microwaving or pressure cooking of the slides prior to antibody application. In both cases, a three step, indirect staining protocol was utilised. Primary monoclonal antibodies were purchased from Oncogene Science, Paris, France, or Chemicon, Harrow, UK (MMP-1, 2, 3, 9, TIMP-1, 2); Sigma (smooth muscle α-actin); or obtained as a gift from Dr W Stetler-Stevenson (MT1-MMP). Biotinylated secondary antibody was purchased from Dako, Ely, UK. Vectastain pre-formed avidin/biotin-horseradish peroxidase conjugated complex (ABC) was purchased from Vector Labs (Burlingame, CA, USA). Diaminobenzidine (DAB) peroxidase substrate was purchased from Merck, Lutterworth, UK.

3.5.1 *Immunohistochemistry*

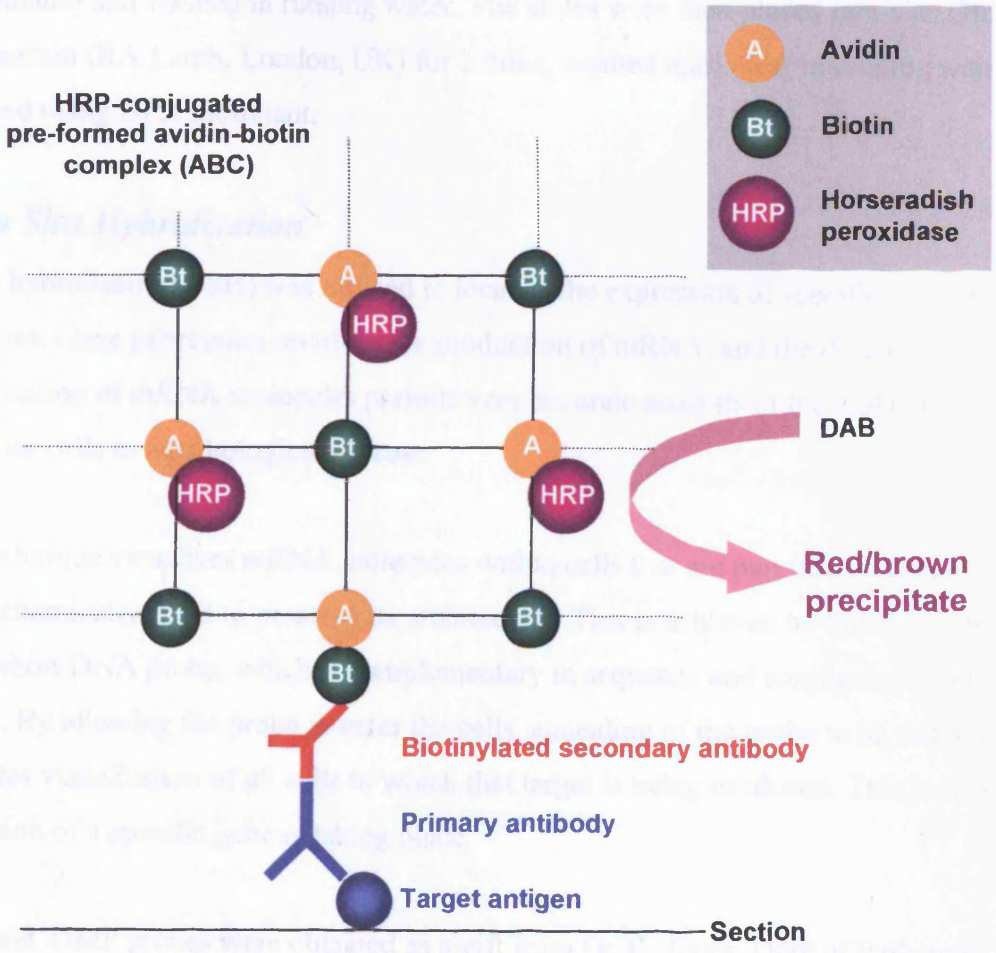
Paraffin wax embedded sections (5μM) were applied to silane coated glass microscope slides and allowed to dry overnight at 37°C. The sections were de-waxed and hydrated by sequential submersion in xylene, 99% ethanol, 95% ethanol and water (1min each), then any intrinsic peroxidase activity blocked by submersion in 6% H_2O_2 for 10 mins. The slides were washed in PBS for 5 mins and blotted carefully. Normal goat serum (diluted 1 in 20; Dako) was applied to the slides to cover the sections and left for 10 mins in a humid chamber, to reduce non-specific antibody binding. The slides were drained and the sections carefully wiped around. 100μl of primary antibody (diluted appropriately in 5% goat serum) was added to the sections and the slides returned to the humid chamber where they were left for 18hours at 4°C. Unbound antibody was removed by two 10min washes in PBS.

The slides were wiped and returned to the humid chamber where 100μl of secondary antibody (diluted 1 in 600 in PBS) was applied to the sections, which were incubated for 30 mins at room temperature. Unbound secondary antibody was removed by two 10 min washes in PBS. The slides were again wiped and returned to the humid chamber, where 100μl of pre-formed

ABC complex was applied to the sections and incubated at room temperature for 30 mins. Excess ABC complex was removed by two 10 min washes in PBS.

DAB substrate (0.05%) was filtered onto the slides and left at room temperature for 5 mins. The slides were then washed briefly in 25mM $CuSO_4$ solution then washed in running water. Cellular nuclei were stained by immersion of the slides in haemotoxylin for 30 secs and washing until clear in running water. The sections were then dehydrated and cleared by sequential immersion in 95% ethanol, 99% ethanol and xylene (1 min each). They were then mounted under glass cover slips using XAM mountant (Merck, Lutterworth, UK). Regions of immunological reactivity were visualised by a dark brown stain, produced where the DAB was oxidised by HRP to produce a red-brown precipitate (Figure 3.1).

Figure 3.1 Schematic representation of the three step indirect immunohistochemical detection system. HRP oxidises DAB to give a visible red/brown precipitate.



3.5.2 *Haemotoxylin And Eosin Staining*

Slide mounted tissue sections were hydrated by passing sequentially through solutions of xylene, 99% ethanol, 95% ethanol and distilled water. They were submerged in haemotoxylin (BDH, Poole, UK) for 4 mins, then washed until clear in running water. They were dipped into 0.5% eosin solution for 5 secs, and again washed until clear in running water. The sections were then dehydrated by reversing the above hydration process, and mounted using DPX mountant (RA Lamb, London, UK).

3.5.3 *Elastin Van Gieson Staining*

Slide mounted tissue sections were hydrated as above. They were submerged in 0.25% potassium permanganate for 5mins, then 0.1% oxalic acid for 5 mins, before washing in running water until clear. The slides were rinsed briefly by submersion in 95% ethanol, then placed into Millers elastin stain (RA Lamb, London, UK) for 45 mins. This was rinsed off in 95% ethanol and washed in running water. The slides were then placed into Van Gieson's counterstain (RA Lamb, London, UK) for 2 mins, washed until clear in running water, then mounted using DPX mountant.

3.6 *In Situ Hybridisation*

In situ hybridisation (ISH) was utilised to localise the expression of specific genes to arterial cell types. Gene expression involves the production of mRNA, and the detection and identification of mRNA molecules permits very accurate analysis of the biochemical input of particular cells to a pathological process.

This technique visualises mRNA molecules within cells that are part of a section of intact tissue, chemically fixed to preserve its architecture. This is achieved by binding to them a novel, short DNA probe, which is complementary in sequence and conjugated to a marker protein. By allowing the probe to enter the cells, annealing of the probe to its mRNA target facilitates visualisation of all cells in which that target is being produced. This indicates that expression of a specific gene is taking place.

MMP and TIMP probes were obtained as a gift from Dr JL Jones, Dept of Pathology, University of Leicester. They had been designed according to sequence data from the

Genbank database. All probes were a cocktail of oligonucleotides, each of 20-30 bases in length, as described below. Anti-digoxin secondary antibody was purchased from Sigma.

Target	Oligonucleotide Sequence	Position
MMP-2	CCT CAT TGT ATC TCC AGA ATT TGT CTC CAG	1704-1733
	ACC GGT CCT TGA AGA AGA AGA TCT CAC CAC	1398-1427
	CTC CAG AAT TTG TCT CCA GC	1703-1722
MT1-MMP	CCC CTT GTA GAA GTA AGT GAA GAC TTC ATC	1590-1619
	CCT CAT CAA ACA CCC AAT GCT TGT CTC CTT	1327-1356
	CAT CCA GAA GAG AGC AGC ATC AAT CTT GTC	1414-1443
	AAT TTG CCA TCC TTC CTC TCG TAG GCA GTG	1286-1315
TIMP-2	ATA TTC CTT CTT TCC TCC AAC GTC CAG CGA	571-600
	AAC TCT ATA TCC TTC TCA GGC CCT TTG AAC	504-533
	GTT CTT CTC TGT GAC CCA GTC CAT CCA GAG	787-816
	GTA CCT GTG GTT CAG GCT CTT CTT CTG GGT	685-714

3.6.1 *Probe Labelling*

To visualise the probes, they were end-labelled with digoxin. This was achieved using a DNA oligonucleotide tailing kit (Boehringer Mannheim, Lewes, UK) according to the manufacturers protocol. This reaction is based on the ability of the enzyme terminal deoxynucleotidyl transferase (Tdt) to add single nucleotides to the 3'-OH terminus of a DNA strand. In this case, digoxin-conjugated dUTP and unconjugated dATP nucleotides were added, typically around 20 bases to each probe molecule. A labelling reaction was made up as follows: 4µl 5x tailing buffer(1M potassium cacodylate; 125mM Tris-HCl; 1.25mg/ml BSA), 5mM $CoCl_2$, 50mM dig-dUTP, 500nM dATP, 1µg oligonucleotide, 50U Tdt enzyme and the reaction made up to 20µl with DEPC water. The reaction was allowed to proceed at 37°C for 1hour.

Following the labelling reaction the probes were purified to remove reaction components and unincorporated nucleotides. They were applied to a QiaQuick oligonucleotide purification spin-column (Qiagen, Crawley, UK). These silica-gel columns bind DNA under high salt conditions and subsequent centrifugation of the loaded column allows passage of impurities. DNA is then recovered by addition of basic elution buffer and further centrifugation. The collected probe solution was stored at -20°C for future use.

3.6.2 *Test Strips*

To test the efficiency of probe labelling, visualisation tests were conducted. This allows an estimation of probe sensitivity as a guide to later application. Probe was diluted to concentrations of 1ng/µl, 100pg/µl, 50pg/µl, 10pg/µl, 1pg/µl and 0.1pg/µl in diluent buffer (900mM NaCl, 90mM trisodium citrate, 20µl 10mg/ml salmon sperm DNA in 1ml DEPC-H_2O). Probes were boiled for 5mins, quenched on ice and collected by centrifugation.

1µl of each probe dilution was applied in a spot to a strip of Hybond C nitrocellulose membrane (Amersham, Bucks, UK) at 1cm intervals and allowed to dry. The membrane was baked at 80°C for 2hrs to fix the probe in place, then re-hydrated by immersion in buffer (100mM maleic acid, 150mM NaCl, pH 7.5) for 1min. The membrane was placed into pre-warmed blocking solution (3% BSA, 0.1% triton X-100 in TBS) at 42°C for 20mins to reduce non-specific secondary antibody binding. The membrane was transferred into a solution of alkaline phosphatase-conjugated anti-digoxin antibody, diluted to 1 in 600 in blocking solution, where it was incubated at room temperature for 30mins. Unbound antibody was removed by two 15min washes in buffer 1. Membranes were soaked in a detection buffer (100mM Tris-HCl pH9.5, 100mM NaCl, 50mM $MgCl_2$) for 2mins, before visualisation of bound antibody was achieved by addition of the membrane to a solution of NBT/BCIP (comprising 0.6mg/ml NBT (stock of 75mg/ml in 30% dimethyl formamide); 0.4mg/ml BCIP (stock of 50mg/ml in dimethyl formamide); 1mM levamisole (stock of 1M in DEPC-H_2O); 100mM tris-HCl, pH 9.5; 50mM $MgCl_2$; 100mM NaCl; made up in DEPC-H_2O), where it was stored in dark conditions for 16hrs. Alkaline phosphatase reacts with BCIP and their product with NBT yields an insoluble blue precipitate. Intensity of staining was compared to a no-probe control to assess sensitivity of each concentration.

3.6.3 *Probe Hybridisation*

Tissue specimens were fixed in 4% paraformaldehyde (PFA) for 24hrs then paraffin wax embedded. Sections were cut to 6µm on a microtome and applied to silane coated slides where they were allowed to dry at 37°C for 24hrs. Tissue architecture was assessed by staining selected slides in haemotoxylin and eosin to visualise cell nuclei and extracellular matrix, as described in Section 3.4.

Sections were de-waxed in xylene then hydrated by sequential immersion in lowering concentrations of alcohol and finally DEPC-water. Slides were immersed in a solution of 2x SSC (300mM NaCl, 30mM trisodium citrate) for 10mins at 70°C, then washed in DEPC-water for 5mins. Slides were drained, 100µl proteinase K (2µl/ml or 5µl/ml of a 1mg/ml stock) added to each section and incubated in a humid chamber at 37°C for 60mins. This enzyme degrades extracellular matrix and cell membranes sufficiently to permit intracellular access of probes, antibodies and detection reagents.

The slides were washed first with cold DEPC-water for 5mins then with cold 0.4% PFA for 20mins. The slides were rinsed in DEPC-water for 5mins then 50µl pre-hybridisation solution (600mM NaCl, 50mM Tris-HCl pH 7.5, 0.1% sodium pyrophosphate, 0.2% polyvinyl-pyrollidone, 0.2% ficoll, 5mM EDTA, 10% dextran sulphate, 30% formamide, 150ng/ml heat denatured salmon sperm DNA) added to each. Slides were incubated for 1hr at 37°C in a humid chamber.

Probe was mixed with 50µl of pre-hybridisation solution at a concentration of 500ng/ml and added directly to the sections, over which coverslips were placed. Access and annealing of the probe was allowed to take place overnight at 37°C in a humid chamber.

Coverslips were carefully removed and the slides washed twice in wash buffer (2x SSC, 30% formamide) for 10mins at 37°C. Slides were submerged in blocking solution for 10mins, and alkaline phosphatase-conjugated anti-digoxin antibody added directly to this at a dilution of 1 in 600. This was allowed to bind for 30mins at room temperature. Unbound antibody was removed by two 5min washes in PBS. Bound probe was visualised by addition of 200µl NBT/BCIP solution to each section, cover slips added and incubation in dark conditions at room temperature. Development of signal was checked periodically and the reaction stopped when appropriate by removal of the coverslips and washing in water for 5mins. Cell nuclei were counterstained in haemotoxylin and sections mounted in Aquamount aqueous mountant (BDH, Poole, UK). Regions of probe annealing were observed as a red-brown precipitate, the reaction product of the reduction of NBT/BCIP by alkaline phosphatase.

Positive control slides were prepared by fixing HT1080 tumour cells onto coverslips and treating them in the same way as tissue sections. Negative controls sections were treated either

without probe, or were pre-treated with RNase H (10mg/ml in 100mM Tris-HCl pH 7.5, 100mM $MgCl_2$) by incubating them at 37°C for 1 hour. ISH was then carried out as described.

3.7 *In Situ Zymography*

In situ zymography was used to confirm data collected from gelatin zymography and *in situ* hybridisation. Its usefulness is apparent when considering that zymography permits the identification, quantification and activity status of MMP production but not its location. In contrast, *in situ* hybridisation defines the cellular site and qualitative information on MMP expression, but gives no information on the quantity or activity status of the enzymes.

In situ zymography permits the visualisation of tissue MMP activity by the ability of the enzymes to cleave quenched fluoroscein-conjugated gelatin, which is applied to tissue sections on microscope slides. Cleavage of the gelatin yields regions of fluorescence when viewed microscopically under mercury vapour lamp illumination. It should be noted however that no information on MMP identity may be derived from this technique.

10μm sections were cut from frozen tissue specimens and applied to silane-coated microscope slides. The slides were submerged in pre-warmed (37°C) fluorosceinated porcine gelatin (Molecular Probes, Leiden, Netherlands; 4μg/ml in 50mM Tris/HCl pH 7.6; 5mM $CaCl_2$; 0.2mM Sodium azide) for 18hours at 37°C. The reverse side of the slide was then wiped dry.

Positive control slides were pre-treated with 200nM phorbol 12 myristate 13 acetate for 30mins at 25°C. Negative controls were prepared by including 10mM 1, 10, phenanthroline within the gelatin solution. Excess pre-treatment solution was drained and the slides carefully rinsed with PBS before submersion.

Visualisation of gelatinolysis was performed by viewing the slides under mercury vapour lamp illumination and appeared as green zones against a dark background. By comparison with white light illuminated sections, the precise location of proteolytic activity was determined.

3.8 *Western Blotting*

Western blots were used to confirm the identity of MMP and TIMP proteins suspected to be present in cell culture medium by inference from zymography experiments. In this technique, proteins are fractionated by SDS-PAGE before being electrophoretically transferred to the surface of a nitrocellulose membrane. Specific proteins are then localised by using a two step immunological detection system, coupled to a chemiluminescent visualisation methodology, which is recorded on x-ray film. MMP/TIMP antibodies were purchased from Oncogene Science (Paris, France) or Chemicon (Harrow, UK); HRP-conjugated rabbit anti-mouse secondary was purchased from Amersham (Little Chalfont, Bucks, UK).

Proteins within culture medium were fractionated by SDS-PAGE. 10% gels were prepared according to the recipes summarised in Table 3.2, from 30%/0.8% acrylamide/bis-acrylamide (Protogel, National Diagnostics, Atlanta, Georgia, USA; resolving buffer (1.5M tris, pH 8.8); stacking buffer (0.5M tris, pH 6.8); 10% SDS; 10% ammonium persulphate (prepared freshly); TEMED; distilled water.

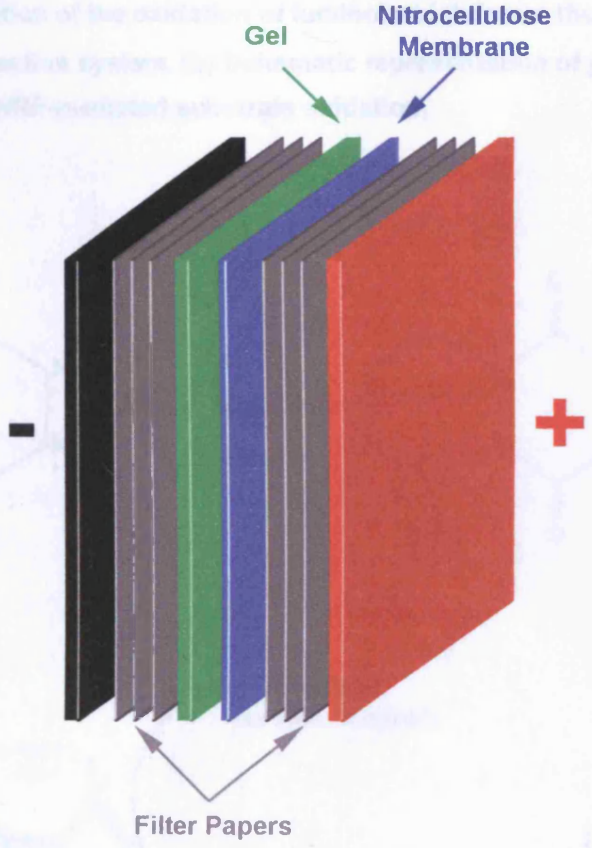
Table 3.2. Contents of polyacrylamide electrophoresis gels.

Reagent	Resolving Gel	Stacking Gel
dH_2O	4ml	6.1ml
30%/0.8% Acrylamide/Bis	3.34ml	1.3ml
Resolving buffer	2.5ml	-
Stacking buffer	-	2.5ml
SDS (10%)	100µl	100µl
Ammonium persulphate (10%)	50µl	50µl
TEMED	5µl	10µl

The electrophoresis tank was filled with tank buffer (25mM tris; 200mM glycine; 3.5mM SDS; pH 8.3). Samples were mixed with an equal volume of reducing loading buffer (62.5mM tris, pH6.8; 1.4M SDS; mM 2-mercaptoethanol; 45% glycerol; 0.025% bromophenol blue) prior to loading onto the gel. All gels were prepared and run in the Mini Protean II electrophoresis system (Bio-Rad, Hemel Hempstead, Herts, UK) at a constant current of 30mA for approximately 4 hours.

Following electrophoresis, the gel was transferred to the Mini Trans-Blot apparatus (Bio-Rad, Hemel Hempstead, Herts, UK) in which the gel was aligned next to an identically sized piece of Hybond ECL nitrocellulose membrane (Amersham, Bucks, UK) and the two sandwiched between three layers of filter paper. The tank was filled with blotting buffer (200mM glycine; 25mM tris; 20% methanol) and 100mA current applied for 18 hours (Figure 3.2).

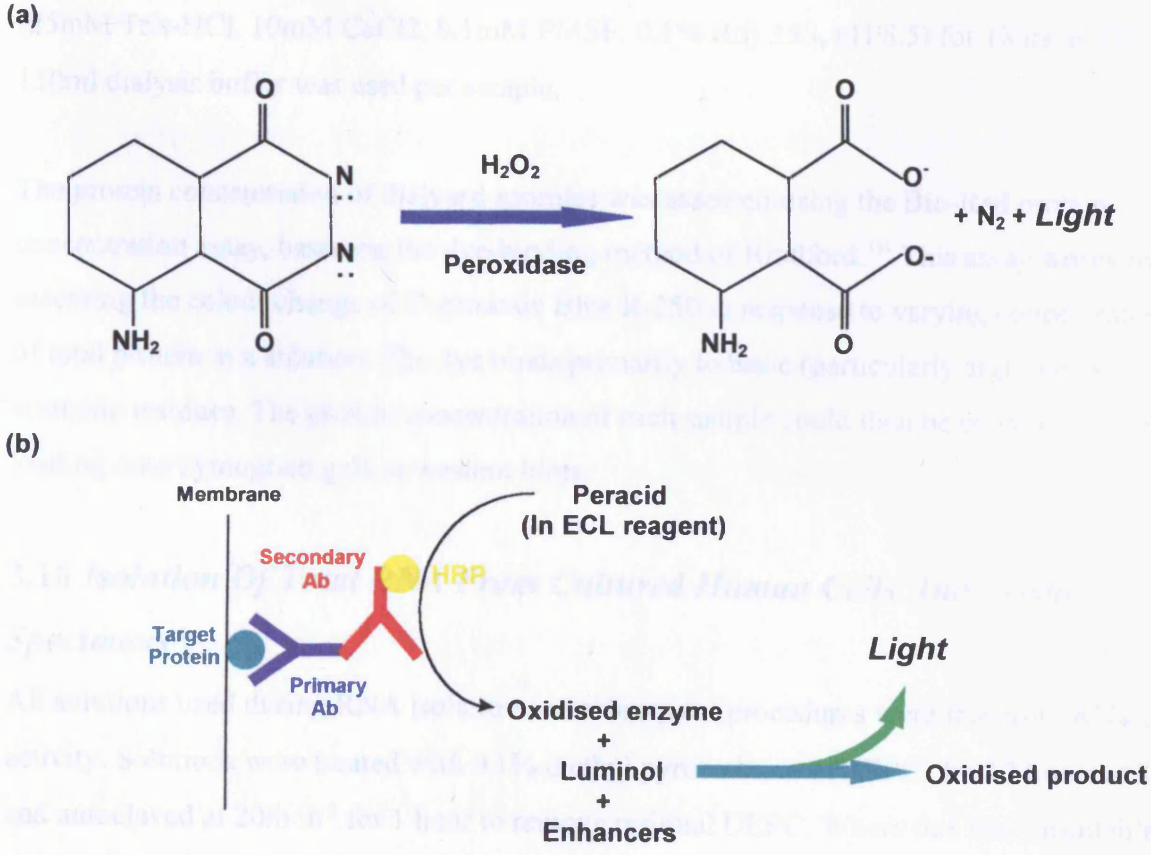
Figure 3.2 Representation of the western blot apparatus used to transfer protein from a polyacrylamide gel to a nitrocellulose membrane. The proteins are negatively charged, and migrate towards the cathode when current is applied.



Following transfer of the proteins to the membrane, non-specific binding of antibody was blocked by its submersion in a solution of 5% milk powder in TBS-T (0.1% Tween 20 in TBS) for one hour at room temperature. The blocking solution was removed and the primary antibody added, diluted to 1 in 500 in TBS-T and incubated for one hour at room temperature. Unbound antibody was removed by thorough washing in TBS-T (3 x 10 mins). Secondary antibody was then added to the membrane, diluted 1 in 2000 in TBS-T, and incubated at room temperature for 30 mins. Unbound antibody was removed by thorough washing in TBS-T (3 x 10 mins).

Visualisation of bands of immunological reactivity was achieved using the ECL detection system (Amersham, Bucks, UK) according to the manufacturers protocol. This is a light-emitting, non-radioactive technique involving the peroxidase-mediated oxidation of a luminol substrate to an excited state (Figure 3.3). This then decays back to ground state via a light emitting pathway, with maximal emission at 428nm. The light produced in this reaction was recorded on X-OMAT-AR film (Kodak, Rochester, New York, USA) following an exposure of approximately 5 minutes.

Figure 3.3 (a) Illustration of the oxidation of luminol which forms the basis of the ECL chemiluminescent detection system. (b) Schematic representation of primary and secondary antibody binding and HRP-mediated substrate oxidation.



3.9 *Homogenisation Of Tissue Specimens*

Tissue specimens were homogenised to analyse their overall MMP content in certain experiments. Samples were rendered down by mechanical disruption and dialysed to extract a specific molecular weight range of the total protein content.

Tissue was weighed accurately, cut into small pieces with a sterile scalpel blade, and 1ml homogenising buffer (2M urea, 50mM Tris-HCl, 1g/L NaCl, 1g/L EDTA, 0.1mM PMSF, 0.1% Brij 35S, pH 7.6) added per 100mg wet weight. The tissue was homogenised thoroughly using a Polytron blender. Homogenates were then centrifuged at 11000rpm for 1hr at 4°C to remove cellular debris.

Supernatants were then collected into 10kDa cut-off Visking tubing (Fisher, Loughborough, UK) which was sealed with plastic clips. Samples were dialysed against dialysis buffer (25mM Tris-HCl, 10mM CaCl2, 0.1mM PMSF, 0.1% Brij 35S, pH 8.5) for 18hrs at 4°C. 150ml dialysis buffer was used per sample.

The protein concentration of dialysed samples was assessed using the Bio-Rad protein concentration assay, based on the dye-binding method of Bradford.[331] This assay works by assessing the colour change of Coomassie Blue R-250 in response to varying concentrations of total protein in a solution. The dye binds primarily to basic (particularly arginine) and aromatic residues. The protein concentration of each sample could then be equalised before loading onto zymogram gels or western blots.

3.10 *Isolation Of Total RNA From Cultured Human Cells And Tissue Specimens*

All solutions used during RNA isolation and subsequent procedures were free from RNase activity. Solutions were treated with 0.1% diethyl pyrocarbonate (DEPC) for 12 hours at 37°C and autoclaved at 20lb in^{-1} for 1 hour to remove residual DEPC. Where this was unsuitable, chemicals were purchased and reserved exclusively for RNA work. All glassware and spatulas were baked at 180°C before use. Plastic-ware was, when necessary, treated with a 3% solution of hydrogen peroxide for 15 mins, then rinsed thoroughly with DEPC-treated water. This work was performed under strict guidelines to maintain RNase free conditions: gloves were worn at all times when handling equipment and reagents, and a designated area of the laboratory was set aside for RNA-based experiments.

Total RNA was isolated from cultured human cells and tissue using Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturers protocol. This reagent is a monophasic solution of phenol and guanidine isothiocyanate, which together cause lysis and disintegration of cells and their component organelles, but maintain the integrity of all nucleic acids. Addition of chloroform to the cell lysate separates the solution into an aqueous and an organic phase, and centrifugation removes all cellular debris, protein and genomic DNA to the lower, organic phase. This leaves RNA exclusively in the aqueous phase which may then be recovered by alcohol precipitation.

Trypsinised cells or homogenised tissue were added to an RNase-free centrifuge tube and 1ml Trizol reagent added per 10^5 cells or 100mg tissue in the suspension. The tube contents were mixed thoroughly by gentle pipette aspiration and left at room temperature for 5 mins. Chloroform was added (200µl per 1ml of Trizol), the solution mixed by vortexing briefly and left at room temperature for 3 mins. The solution was then centrifuged at 13000g for 15 mins, and the upper, aqueous phase collected and transferred to a fresh RNase free tube. The RNA was precipitated by addition of 500µl propan-2-ol per 1ml Trizol used, which was mixed by inversion and left at room temperature for 10mins. The solution was centrifuged at 13000g for 15mins to pellet the RNA, the pellet washed in 75% ethanol, air-dried briefly and resuspended in RNase-free water. The concentration of the isolated RNA was determined spectrophotometrically, by using a Gene-Quant analyser (Pharmacia, St Albans, UK).

3.11 *Reverse Transcription Polymerase Chain Reaction (RT-PCR)*

RT-PCR was carried out to confirm the expression of MMP and TIMP mRNAs. Reverse transcription was performed using AMV-RT enzyme and oligo dT_{15} primer (Promega, Madison, WI, USA) as directed in the enzyme literature. Each reaction was made up of 5µl AMV-RT buffer (250mM Tris-HCl pH 8.3; 250mM KCl; 50mM $MgCl_2$; 50mM DTT; 2.5mM Spermidine), 200µM each dNTP, 0.5µg oligo dT_{15} primer, 40U RNasin, 100ng total RNA, 10U AMV-RT enzyme and the reaction volume made up to 20µl with DEPC-H_2O. All reactions were overlaid with mineral oil and incubated at 42°C for 60mins.

Amplification of specific sequences was performed using standard PCR methodology and novel primers, obtained as a gift from Dr JL Jones, as described below.

MMP-2 Forward	ATT GAT GCG GTA TAC GAG GC	350bp Product
MMP-2 Reverse	GGC ACC CTT GAA GAA GTA GC	
MT1-MMP Forward	TGC CCA ATG GAA AGA CCT AC	315bp Product
MT1-MMP Reverse	TGA TGA TCA CCT CCG TCT CC	
TIMP-2 Forward	AAC GAC ATT TAT GGC AAC CC	250bp Product
TIMP-2 Reverse	ACC TGT GGT TCA GGC TCT TC	
GAPDH Forward	AGA ACA TCA TCC CTG CCT C	350bp Product
GAPDH Reverse	GCC AAA TTC GTT TGC ATA CC	
β Actin Forward	TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA	661bp Product
β Actin Reverse	CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG	

PCR reactions were made up of 2μl 10x PCR buffer (500mM KCl; 100mM Tris-HCl pH 9.0; 15mM $MgCl_2$; 1% Triton X-100), 200μM each dNTP, 10pmol each primer, 1μl RT product, 5U *Taq* polymerase and made up to a total volume of 20μl with dH_2O. Negative controls lacked cDNA template or *Taq* polymerase. A positive control was included, using a primer set for the GAPDH or β actin gene (Stratagene, La Jolla, CA, USA).

All reactions were overlaid with mineral oil and thermal cycling was carried out using the following parameters: 1 cycle of 95°C for 5mins, 59°C for 1min, 72°C for 1min; 29 cycles of 95°C for 1min, 59°C for 1min, 72°C for 1min, 1 cycle of 95°C for 1min, 59°C for 1min, 72°C for 10mins. All reaction products were analysed on a 1% agarose gel, stained with ethidium bromide and photographed under 254nm ultraviolet illumination.

3.12 *Northern Blotting*

Northern blotting was used to compare the level of expression of MMP and TIMP genes. In this technique, total RNA is fractionated electrophoretically using an agarose/formaldehyde gel to maintain the RNA in a denatured state. Denaturation of the RNA permits even separation of fragments, and facilitates hybridisation of a complementary probe when transferred to a solid support. Following electrophoresis the RNA is transferred to the surface of a nylon membrane by capillary transfer, where radiolabelled oligonucleotide probes are applied. Hybridisation of these probes to their complementary target allows visualisation of specific mRNAs by detection of radioactive decay on an x-ray film. The amount of probe hybridised gives a relative measure of the amount of mRNA target within a total RNA sample.[332]

3.12.1 *Electrophoresis*

A 1% agarose/formaldehyde gel was prepared by melting 1g of Seakem GTG agarose (National Diagnostics, Atlanta, USA) in 82ml MESA buffer (40mM 3-(N-morpholino) propanesulphonic acid, pH 7.0; 10mM sodium acetate; 1mM EDTA), adding 18ml formaldehyde inside a fume hood, mixing and pouring into the bed of a 15x10cm gel kit with comb inserted. When set, the comb was removed and the gel submerged in fresh MESA buffer. Samples were prepared by mixing 5.5µl formaldehyde, 15µl formamide, 1.5µl 10x MESA buffer, DEPC-water and total RNA to make up to 30µl. This was then heated to 70°C for 15mins to denature the RNA, quenched on ice and added to 1µl loading buffer (50% glycerol; 1mM EDTA, pH 8.0; 0.025% bromophenol blue; 0.025% xylene cyanol FF). The samples were loaded into the wells of the gel and 30mA current applied until the first dye front reached the end of the gel. The gel was then soaked in three changes of DEPC-water and in 20x SSC (3M trisodium citrate; 3M sodium chloride) for 30mins.

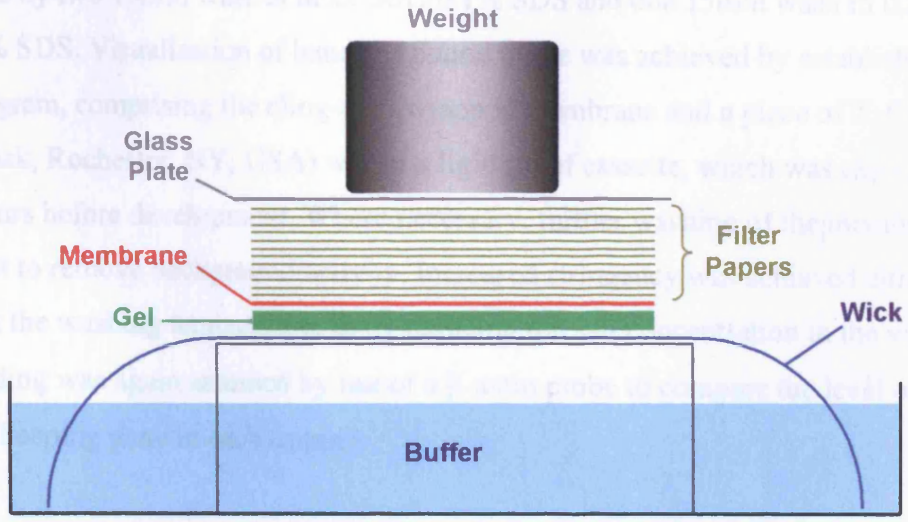
3.12.2 *Capillary Blotting*

A capillary transfer apparatus was set up by placing a Whatman 3MM paper wick over a glass plate suspended above a tank filled with 20x SSC buffer. The wick was immersed at both ends and allowed to become wet over its entire surface. The gel was placed onto the wick, covered with an identically sized piece of Hybond N nylon membrane (Amersham, Little Chalfont, Bucks, UK) and topped with 15 sheets of Quickdraw blotting paper, also cut to size. The whole stack was covered with a small glass plate onto which a 0.25kg weight was placed to promote capillary action (Figure 3.4). Transfer was allowed to proceed for 18 hours.

Buffer is drawn up through the gel and membrane and into the blotting paper. Thus, RNA is carried from the gel onto the membrane, where it is bound. Once complete, the stack was disassembled and the position of the lanes marked. The RNA was fixed in place on the membrane by baking at 80°C for 2 hours. The membrane was then cut into strips according to the lanes of RNA loaded, using a sterile scalpel blade. At this stage specific lane strips were stained to directly visualise their RNA content (for example RNA standards) by soaking them in a solution of 0.03% methylene blue in 0.3M sodium acetate, pH 6.2, for 60 secs then washing in DEPC-water. RNA appeared as blue bands on the white membrane. This was used

to equalise loading between lanes, by assessing the intensity of staining of 18S and 28S rRNA bands.

Figure 3.4 Representation of northern blot apparatus. A buffer flow is set up when the dry filter papers draw liquid up through the wick and gel by capillary action. RNA molecules are carried from the gel onto the nylon membrane by the buffer, where they bind.



3.12.3 *Probe Hybridisation*

Membrane strips were placed into hybridisation tubes (Hybaid) with 10ml QuickHyb solution (Stratagene, La Jolla, CA, USA, containing 100µg herring sperm DNA) and placed into a rotisserie oven at 65°C for 15 mins. Pre-hybridisation is necessary to improve the conditions for specific probe annealing, by reducing non-specific interaction with the membrane itself.

Radioactively labelled probes were prepared using an RT-PCR-based methodology. Initial amplification of an MMP/TIMP fragment was carried out as described in Section 3.10, using cDNA synthesised from HT-1080 cell total RNA. A second PCR reaction was then carried out, using the first PCR product as a template. This second reaction contained only the reverse primer, thus amplifying in one direction only to yield a product complementary to the relevant mRNA sequence. Furthermore, one dNTP was replaced by a radiolabelled version, to generate a high activity probe. A typical probe labelling reaction was made up as follows: 1µl 10x PCR buffer (as before); 10ng cDNA template; 10pmol reverse primer; 200µM dATP, dGTP, dTTP; 5µl (α-^{32}P) dCTP (3000Ci/mmol, 10mCi/ml, NEN, Boston, MA, USA); 5U *Taq* polymerase

and made up to 10µl with dH_2O. The reaction was overlaid with mineral oil and thermal cycling carried out according to the parameters stated in Section 3.10. The PCR product was mixed with 1ml pre-heated pre-hybridisation solution and added directly to the hybridisation tube. The probe was allowed to anneal for 2 hours at 65°C in the rotisserie oven.

Following hybridisation, unbound and non-specifically bound probe was removed from the membrane by two 15min washes in 2x SSC/0.1% SDS and one 15min wash in 0.2x SSC/0.1% SDS. Visualisation of bands of bound probe was achieved by establishing an autoradiogram, comprising the cling-film-wrapped membrane and a piece of X-OMAT-AR film (Kodak, Rochester, NY, USA) within a light proof cassette, which was exposed for at least 4 hours before development. Where necessary, further washing of the membrane was carried out to remove background activity. Increased stringency was achieved either by increasing the washing temperature, or by reducing the salt concentration in the wash buffer. Equal loading was again assessed by use of a β-actin probe to compare the level of expression of a housekeeping gene in each sample.

3.13 *Statistical Analysis*

All data calculations, statistical analyses and graph preparations were carried out using the Prism 2.01 software package (Graphpad Software, San Diego, USA). Non-parametric tests were employed, and in all instances a p value of less than 0.05 was assumed to be significant. Wilcoxon's matched pairs test was applied to paired data, such as zymogram or northern densitometry data. Mann-Whitney rank sum analysis was used when two unpaired groups of data were compared, such as ELISA data. Kruskall Wallis analysis was used when more than two groups of data were compared. Data was expressed where appropriate as median values with 95% confidence intervals and p value.

SPECIAL NOTE

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CHAPTER FOUR

Results:

Histological Studies

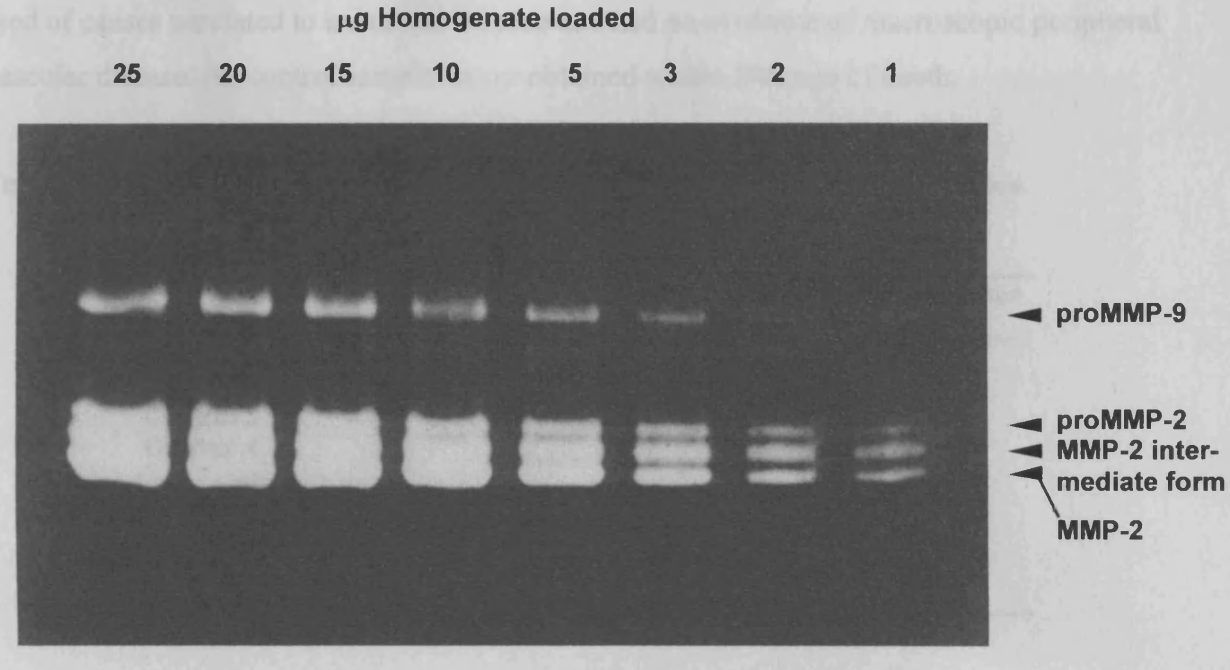
4.1 *Introduction*

A number of previous investigations have described the histological features of AAAs and in some cases have revealed the site of production of MMPs, TIMPs and other molecules associated with ECM degradation. Preliminary studies on homogenised aneurysmal aortic wall demonstrated the presence of latent and active MMP-2 and MMP-9 (Figure 4.1), confirming the presence of these enzymes in the diseased artery. Other groups have used immuno-histochemistry and *in situ* hybridisation to examine enzyme and inhibitor production.[190,193-197] However, no previous study has specifically examined the role of the SMC in the aetiology of AAAs, instead concentrating on the inflammatory cells as a major influence on ECM catabolism. Furthermore, no studies on the role of MT1-MMP in AAAs have been reported. The function of this MMP, as an activator of MMP-2, suggests that it may contribute indirectly to the destruction of elastin which is characteristic of AAAs.

Previous work has stressed the importance of MMP-9 in the elastolytic process of AAAs, a product mainly of infiltrating inflammatory cells. As well as reiterating these findings, the work presented in this chapter aimed to closely examine the source of the MMP-2/MT1-MMP/TIMP-2 enzyme-inhibitor system in aneurysmal aortic tissue, and to compare this to control, age-matched aorta. As a major contributor to the degradation of ECM components, and elastin in particular, the location and activity of this enzyme system may be very important in the development of AAAs. Immunohistochemistry and *in situ* hybridisation were used to locate the source of MMPs and TIMPs at the cellular level.

To further examine the activity of MMP-2 and other proteases, *in situ* zymography was utilised. This relatively new technique has rarely been reported, particularly as applied to vascular pathologies, and represents entirely novel data in research on AAAs. The present study attempted to reveal the presence and location of elastolytic MMPs, their activators and inhibitors, and to consider the relevance of this information as it relates to aneurysm formation.

Figure 4.1 Gelatin zymogram of whole tissue homogenates of human aneurysmal aorta, illustrating the presence of latent MMP-9, proMMP-2 and active forms of MMP-2. This evidence supported the hypothesis that locally produced and activated MMP-2 may be responsible for elastin damage.



Summary Of The Aims Of This Chapter

- To examine the role of the SMC in AAAs as a potential source of elastolytic MMP activity
- To use immunohistochemistry to define the source of MMPs and TIMPs in control and aneurysmal aortic tissue
- To use *in situ* hybridisation to define the source of the MMP-2/MT1-MMP/TIMP-2 enzyme-inhibitor system in control and aneurysmal aortic tissue
- To use *in situ* zymography to examine the activity status of the proteases present in control and aneurysmal aortic tissue

4.2 *Structural Histological Studies*

The histological studies described in the present chapter were performed using tissue derived from the patients listed in Table 4.1. Aneurysm tissue was obtained during elective surgical repair from the aortic wall 2cm distal of the left renal vein, as described in materials and methods. Control tissue was obtained from post-mortem examination of cadavers who had died of causes unrelated to aneurysm disease and had no evidence of macroscopic peripheral vascular disease. All control samples were obtained within 24hours of death.

Table 4.1 List of patients from whom tissue was obtained for histological studies.

	Sex	Age	Aortic Diameter (cm)
Control 1	M	63	<2.5
Control 2	M	59	<2.5
Control 3	F	67	<2.5
Control 4	M	66	<2.5
Aneurysm 1	M	67	7.0
Aneurysm 2	M	69	6.5
Aneurysm 3	M	73	8.0
Aneurysm 4	M	67	7.5

To maintain consistency in figures, sections were illustrated with the adventitial layer on the left side, with the media the main focus of attention. Figure 4.2 shows haemotoxylin and eosin (H&E) stained sections of control and aneurysmal aorta. Contrasts were immediately obvious, with the control tissue being highly organised with intact intimal, medial and adventitial layers identifiable. SMC nuclei were visible in the medial layer, as were the nuclei of fibroblasts and sparse inflammatory cells in the adventitia. The aneurysm tissue was disorganised and comparatively acellular, with few SMCs and a prominent inflammatory infiltrate which localised to dense patches within the media and adventitia. Elastin Van Gieson (EVG) stained sections, shown in Figure 4.3 further revealed the degenerative changes characteristic of AAA. The control aorta exhibited intact elastin and collagen fibres, with prominent SMCs all within the medial elastic lamellae. In contrast, the AAA tissue had profoundly fragmented elastin, visibly reduced collagen fibre and SMC numbers and a thinned medial layer, which was not distinctly identifiable. Medial and adventitial inflammatory cells were also visible, as was the thickened adventitia, which contained numerous vasa vasorum.

Figure 4.2 **Haemotoxylin and eosin stained sections of (a) control aorta and (b) aneurysmal aorta (x80 and x160). Micrographs illustrate the organisation and cellularity of the respective sections, demonstrating the dense SMC content of the control aortic media, compared to the sparse SMC content of the AAA tissue. Also illustrated is the presence of inflammatory cells in the AAA tissue, which are absent from control aorta.**

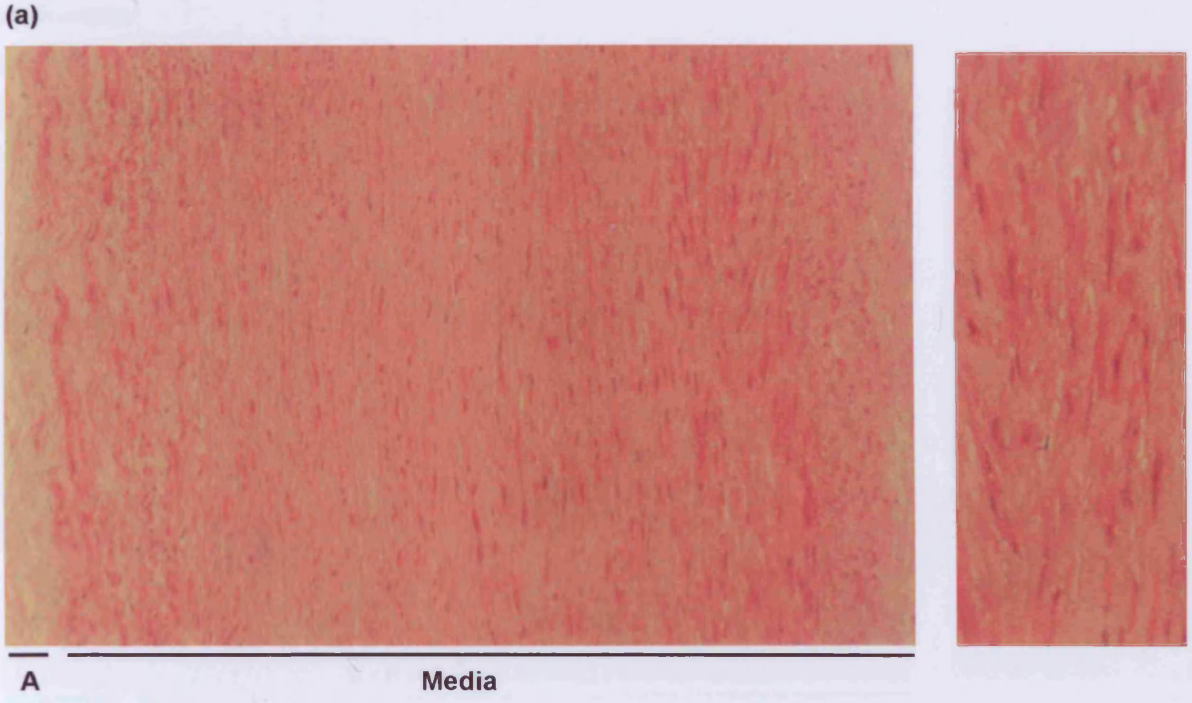
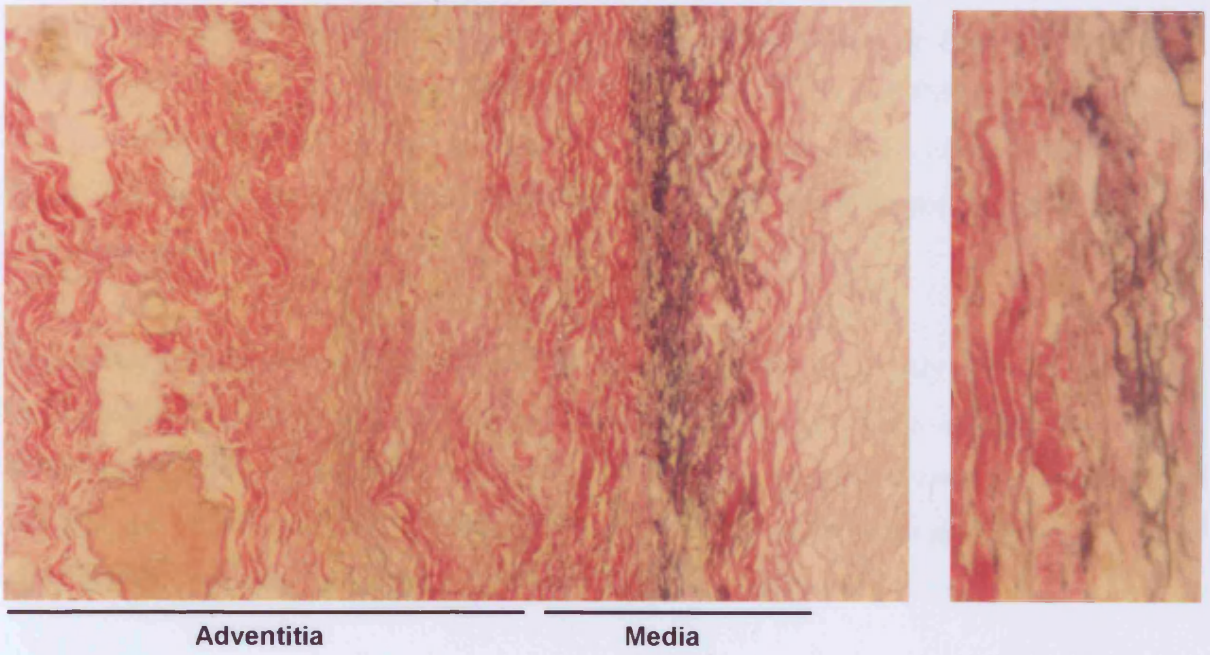


Figure 4.3 **EVG stained sections of (a) control aorta and (b) aneurysmal aorta (x80 and x320). These micrographs illustrate the major structural and ECM components of the aortic media, including the elastin (stained black), collagen (stained pink) and SMCs (stained yellow). Control aorta contained intact and organised lamellae within a thick media. This contrasted strongly with AAA tissue, in which the layers were disorganised and elastin fragmented. The medial layer was thinned and SMC numbers reduced, with inflammatory cells (stained yellow) prominent.**

(a)

(b)



4.3 *Immunohistochemical Studies*

More specific detail of these changes was visible when staining immunohistochemically for specific cell types. The reduction in SMC number in AAA was demonstrated by the use of an anti-smooth muscle actin antibody (Figure 4.5). A broad band of reactivity, associated with the medial elastic lamellae, was seen in the control aorta. AAA tissue showed markedly reduced reactivity in the media, which was thinned. The influx of inflammatory cells was also clearly defined by the use of an anti-leukocyte antibody (Figure 4.6). Little or no reactivity was detected in the control aorta, in contrast to the AAA tissue, where leukocytes were present in dense patches within the medial and adventitial layers. These leukocytes may represent both macrophage and lymphocyte activity, and the density of their appearance perhaps suggests the formation of lymphoid follicles, which may imply an ongoing cellular immune response.

Other groups have attributed much of the ECM degrading activity present in AAA tissue to the inflammatory cells. The present work confirmed that infiltrating leukocytes produced MMP-9, along with MMP-1, 2, MT1-MMP and TIMP-1 and 2, but not MMP-3 (Figures 4.7 to 4.13). However, other cells were shown to be capable of producing some of the same MMPs described above, in particular the medial SMCs. In the media of control aorta, only MMP-2 and small concentrations of MT1-MMP were detected, along with TIMP-1 and 2. These were located diffusely within the medial layer, but were particularly concentrated towards the adventitial aspect and to a lesser extent in the adventitial layer. In AAA tissue, the relative lack of SMCs revealed a reduction in MMP reactivity, although they were shown to produce MMP-1, 2, 9 and MT1-MMP and TIMP-1 and 2. Again, no MMP-3 was detected in the vicinity of the SMCs. Figures 4.14 and 4.15 summarise the association between SMCs and MMP-2 along with leukocytes and MMP-9, defining more clearly the main sources of these two enzymes *in vivo*.

In general, the immunohistochemistry results confirmed work previously reported, but also demonstrated for the first time that the medial SMCs in both control and aneurysmal aortic tissue were capable of producing MT1-MMP. These data also demonstrated the production of MMP-2 and TIMP-2, and the co-localisation of these three components may have considerable implications for the role of MMP-2 in the initiation of AAAs.

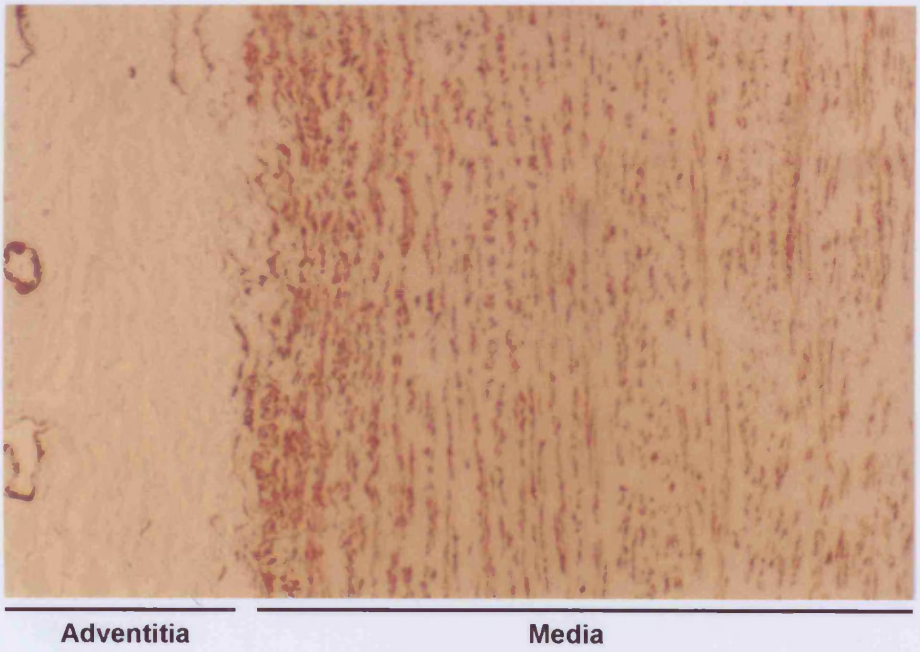
Figure 4.4 **Negative control from immunohistochemical staining of (a) control aorta and (b) aneurysmal aorta (x80). Negative controls were prepared either by treating without primary antibody, or with an alternative, irrelevant primary antibody, such as mouse IgG. The sections below illustrate the lack of reactivity, suggesting little background signal was generated by the secondary detection steps of the protocol.**

(a)

(b)

Figure 4.5 Immunohistochemical staining of (a) control aorta and (b) aneurysmal aorta using an anti-smooth muscle α-actin antibody (x80). Control aorta exhibits many evenly distributed SMCs within the medial layer, which are absent from the adventitia (on left of figure). AAA tissue has very few SMCs, which are present in a thin band, correlating with remaining elastin as seen in Figure 4.2b.

(a)



(b)

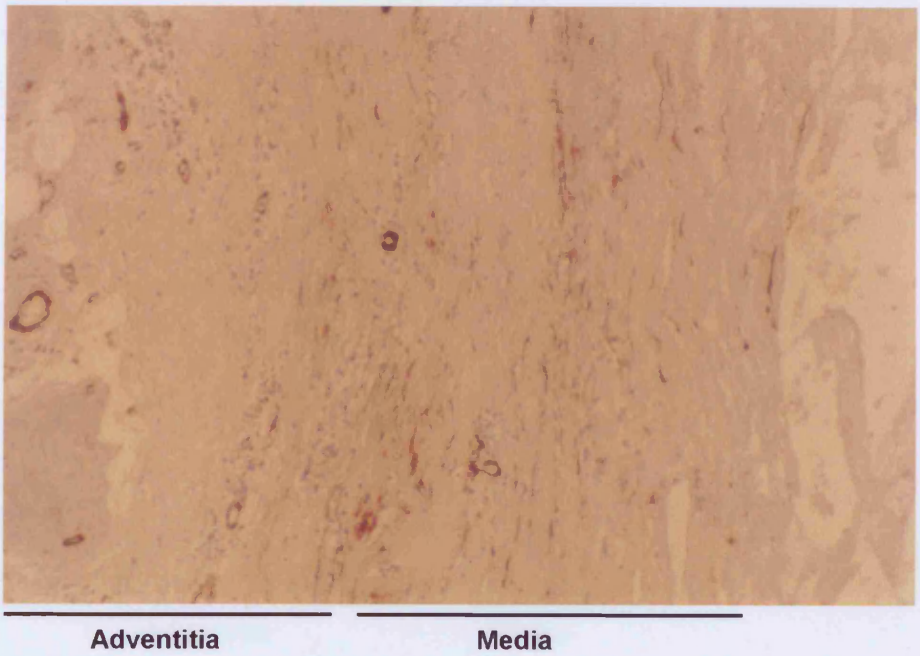


Figure 4.6 **Immunohistochemical staining of (a) control aorta and (b) aneurysmal aorta using an anti-leukocyte antibody (x80). Control aorta contained very few leukocytes, which were scattered through the adventitial layer. In contrast, AAAs contained dense patches of leukocytes, mainly in the adventitial region of the vessel wall, but also within the media, as illustrated below.**

(a)

(b)

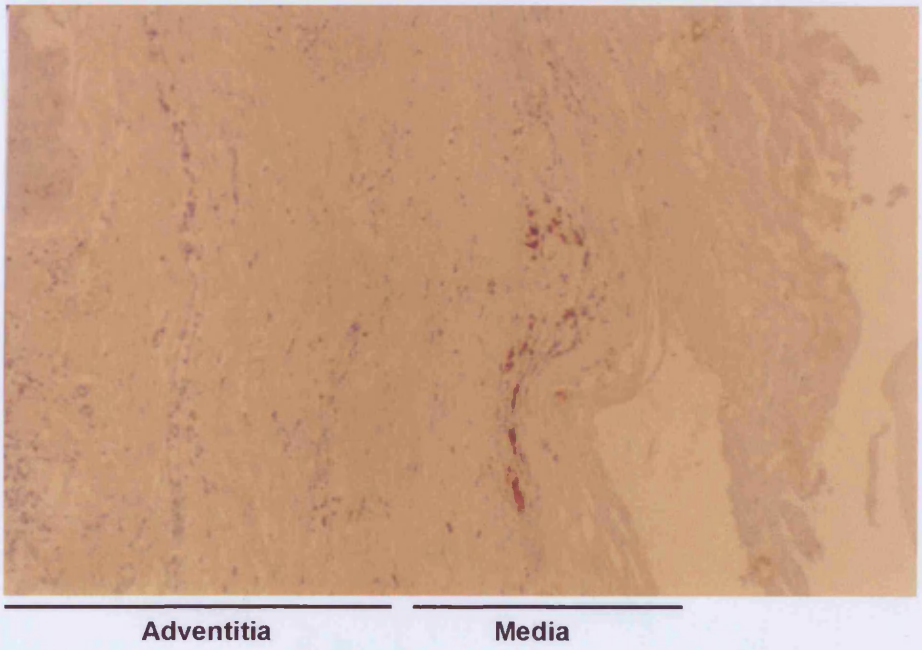
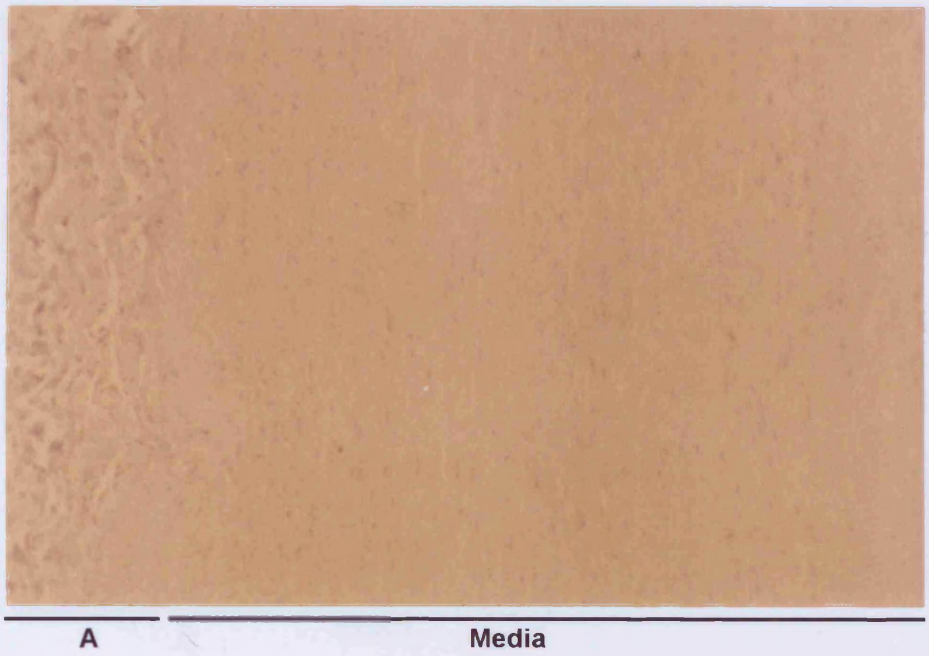


Figure 4.7 **Immunohistochemical staining of MMP-1 in (a) control aorta and (b) aneurysmal aorta (x80). MMP-1 was absent from control aorta. However, some strong reactivity was detected in AAAs, which appeared to co-localise with both inflammatory cells and to a lesser extent with SMCs, throughout the vessel wall.**

(a)



(b)

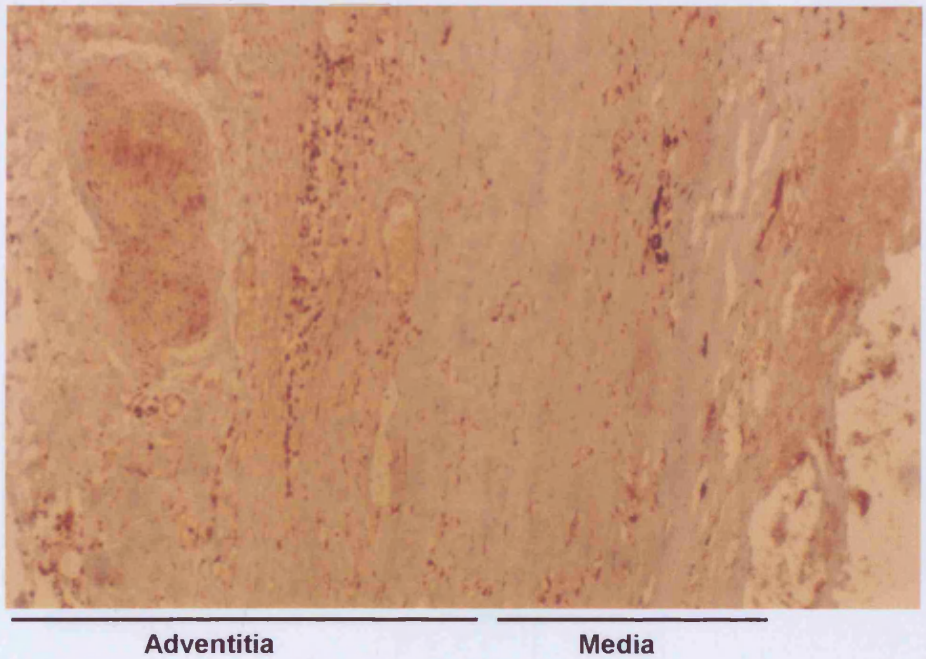


Figure 4.8 **Immunohistochemical staining of MMP-2 in (a) control aorta and (b) aneurysmal aorta (x80 and x320). Strong reactivity in control aorta co-localised to SMCs in the medial layer, and was strongest towards the adventitial aspect. In AAAs the MMP-2 reactivity was widespread, being detected in the vicinity of both remaining SMCs and inflammatory cells.**

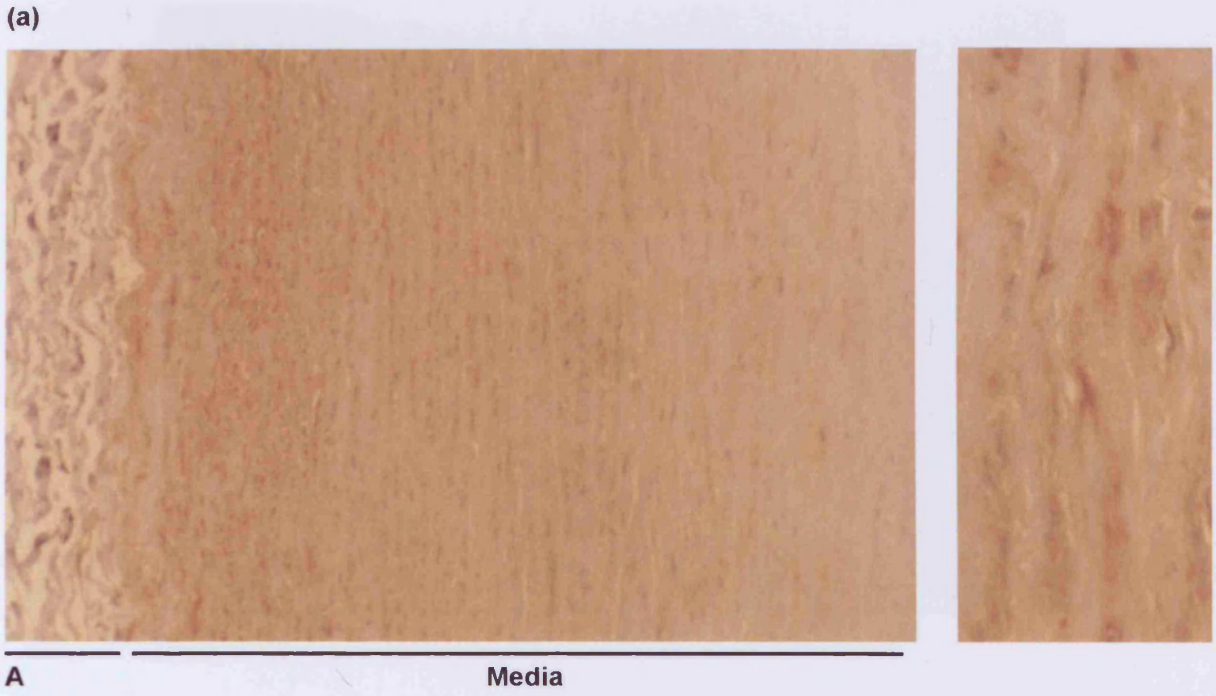
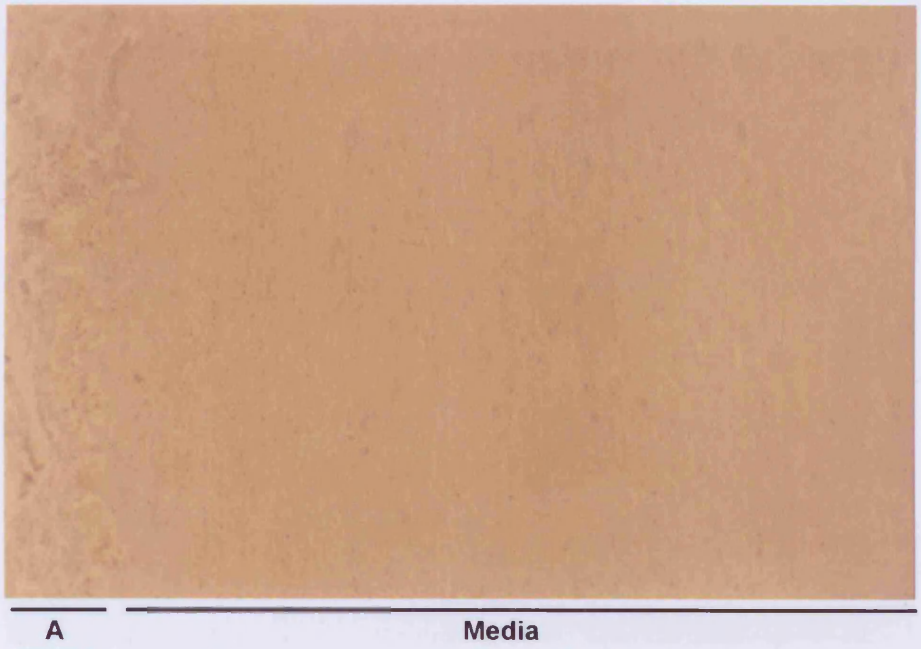


Figure 4.9 **Immunohistochemical staining of MMP-3 in (a) control aorta and (b) aneurysmal aorta (x80). MMP-3 was absent from control aorta and reactivity was extremely low in AAAs, where it was associated with leukocytes.**

(a)



(b)

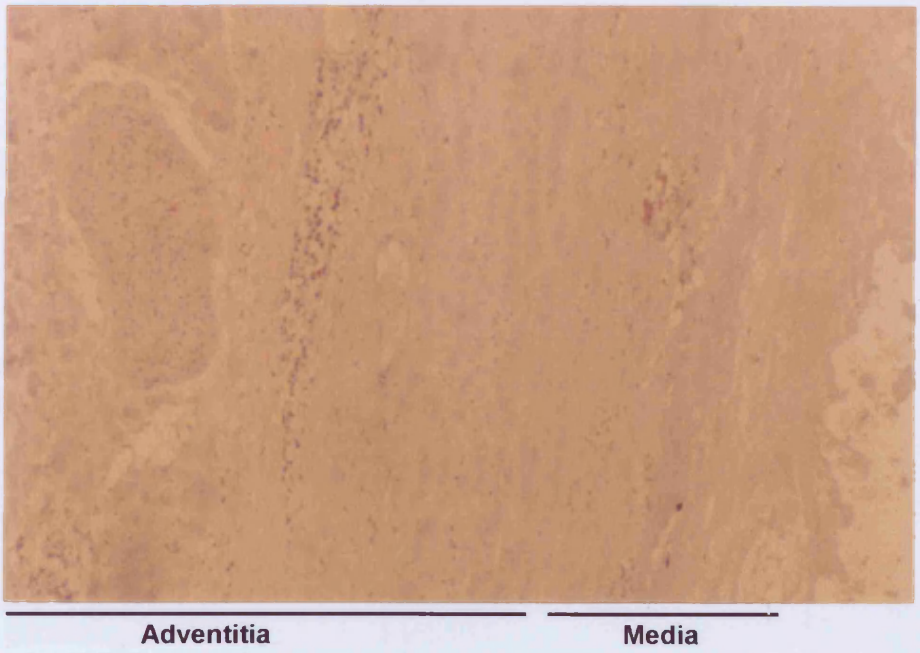


Figure 4.10 **Immunohistochemical staining of MMP-9 in (a) control aorta and (b) aneurysmal aorta (x80). Reactivity with this antibody was low in control aorta, although some MMP-9 was associated with medial SMCs. AAAs contained very high reactivity, correlating with both SMCs and leukocytes, but this may reflect a high level of background staining.**

(a)

(b)

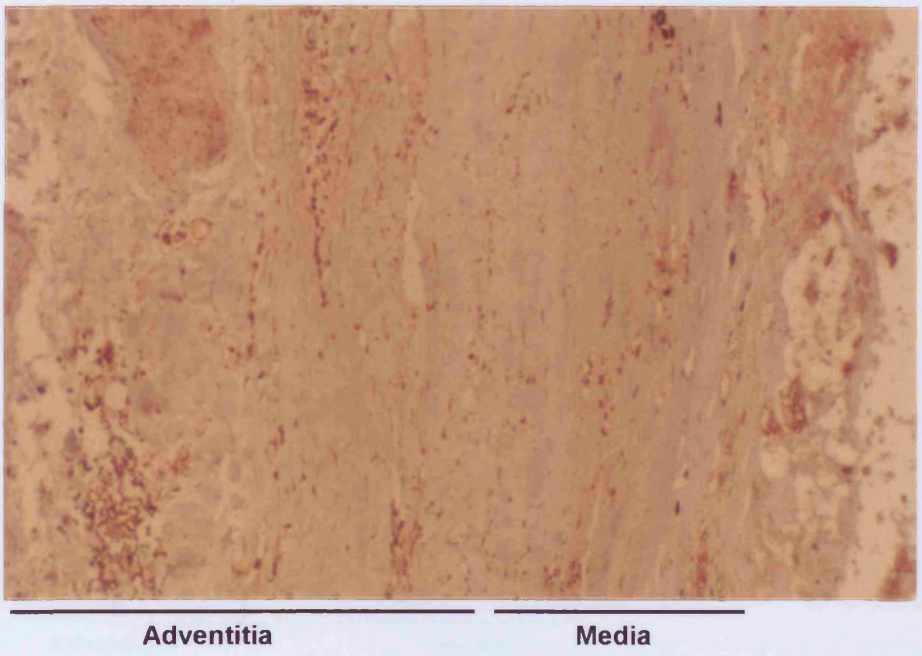


Figure 4.11 **Immunohistochemical staining of MT1-MMP in (a) control aorta and (b) aneurysmal aorta (x80 and x320). Control aorta contained moderate levels of reactivity and the source of MT1-MMP production appeared to be the medial SMCs. Similarly, MT1-MMP was present in small amounts in AAAs, again co-localising with SMCs along with leukocytes.**

(a)

(b)

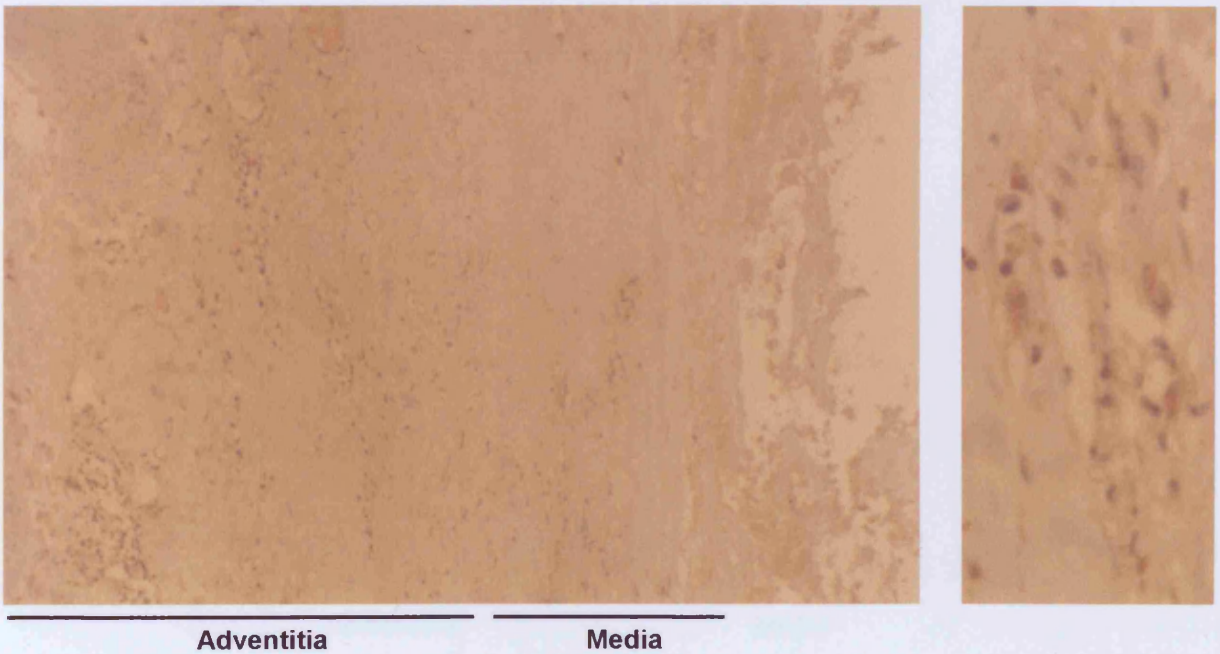


Figure 4.12 **Immunohistochemical staining of TIMP-1 in (a) control aorta and (b) aneurysmal aorta (x80). TIMP-1 was present in a diffuse pattern throughout the medial layer of control aorta, appearing to be a product of SMCs. In AAAs, TIMP-1 was also widely detected, localising to most regions of cellularity, including SMCs and leukocytes.**

(a)

(b)

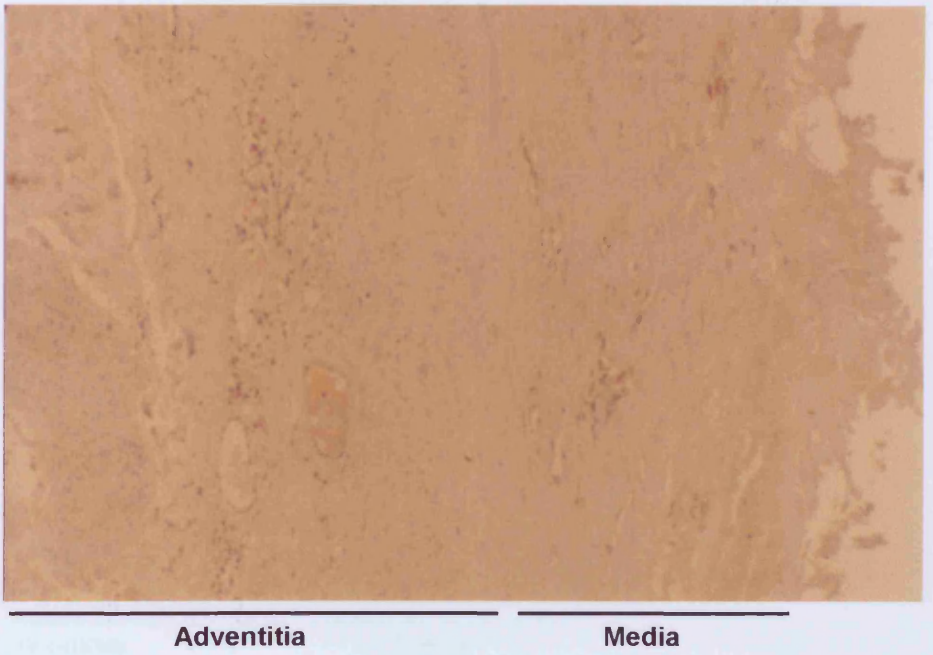


Figure 4.13 **Immunohistochemical staining of TIMP-2 in (a) control aorta and (b) aneurysmal aorta (x80 and x320). Like TIMP-1, TIMP-2 was present in a diffuse pattern throughout the medial layer of control aorta, localising to the SMCs. TIMP-2 also appeared to be produced by both SMCs and leukocytes in AAAs.**

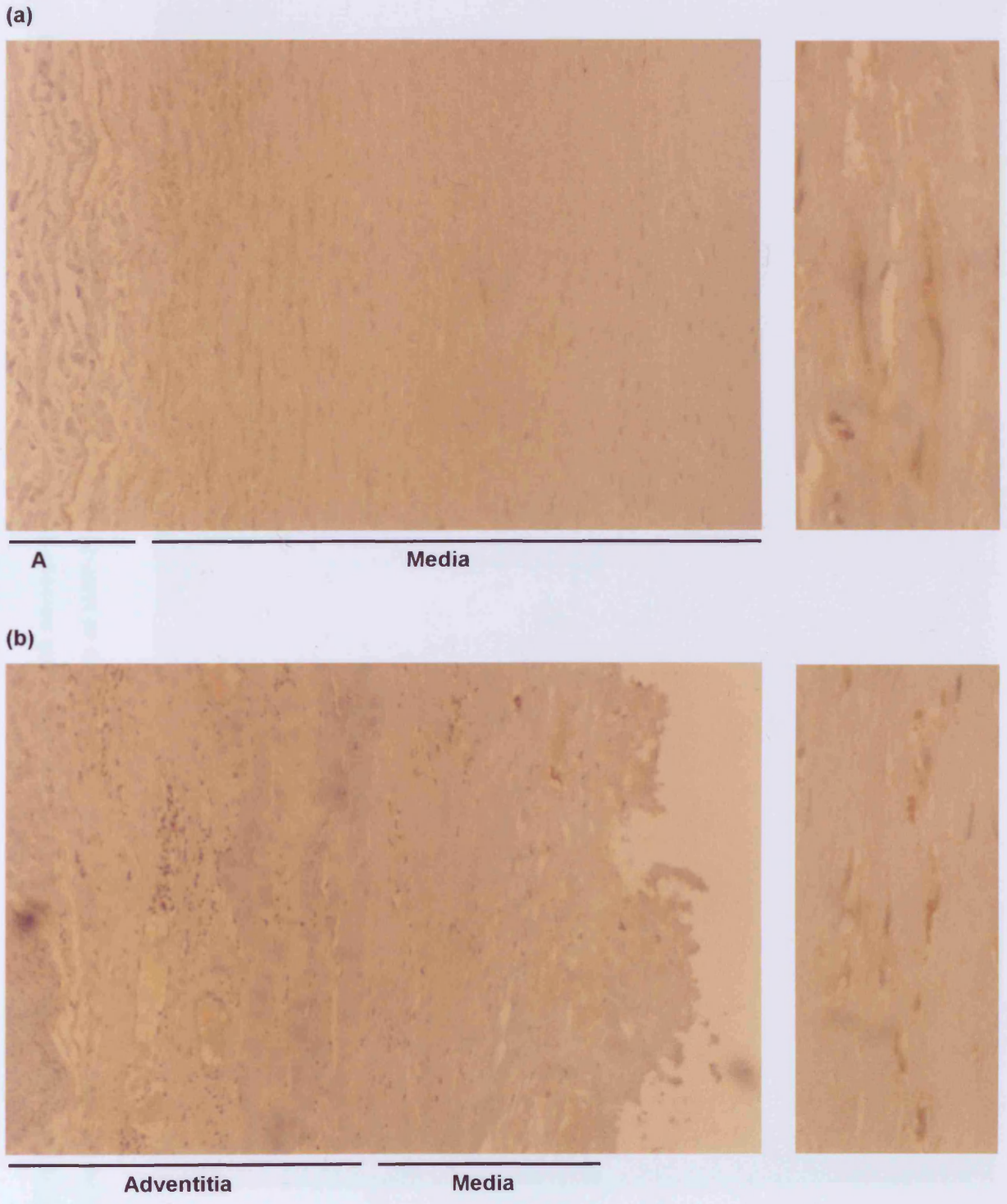


Figure 4.14 Immunohistochemical staining of control aorta, with smooth muscle α actin (SMA), MMP-2, macrophage (CD68) and MMP-9 antibodies. This configuration demonstrated clear co-localisation of MMP-2 with SMCs and MMP-9 with macrophages.

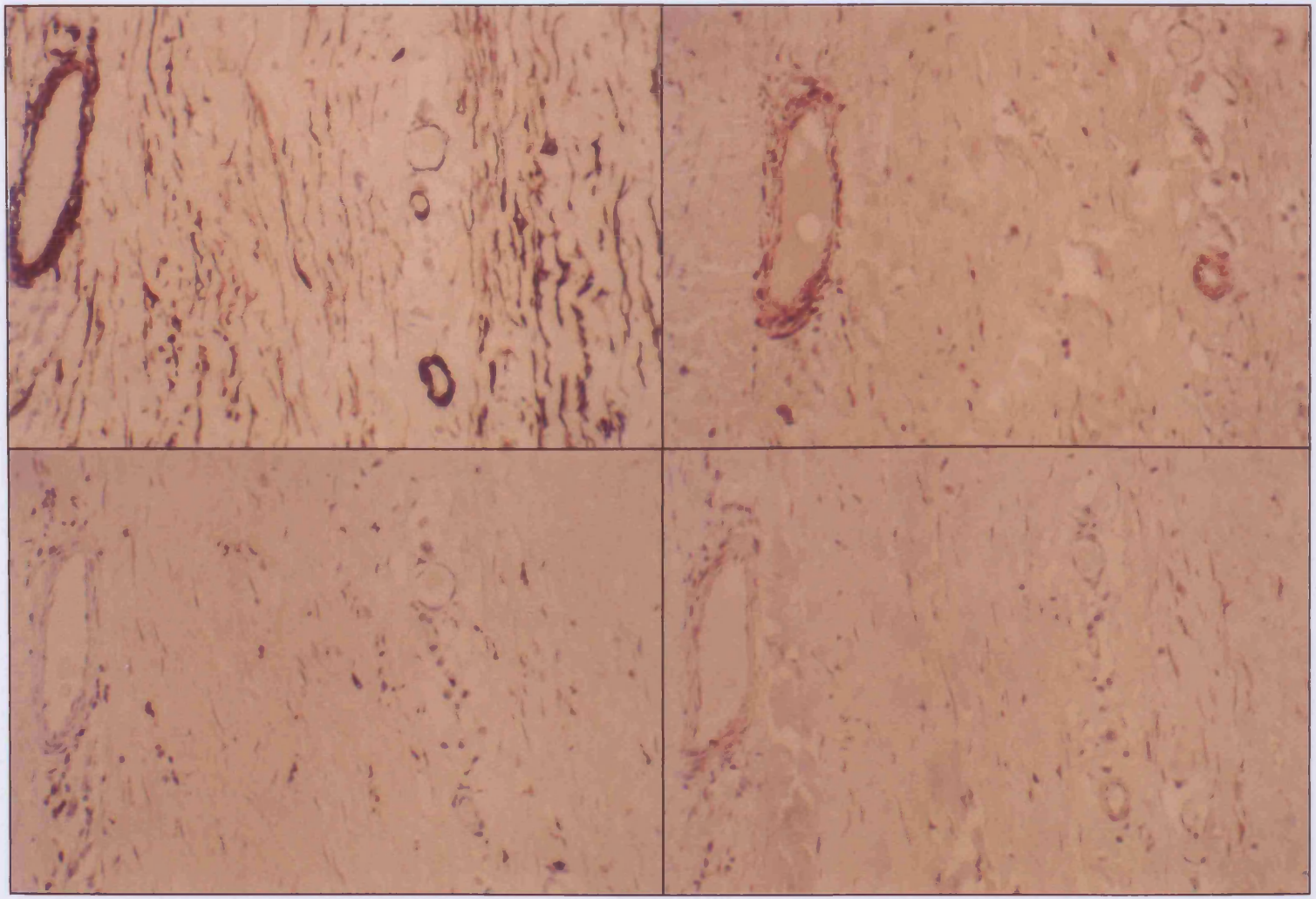
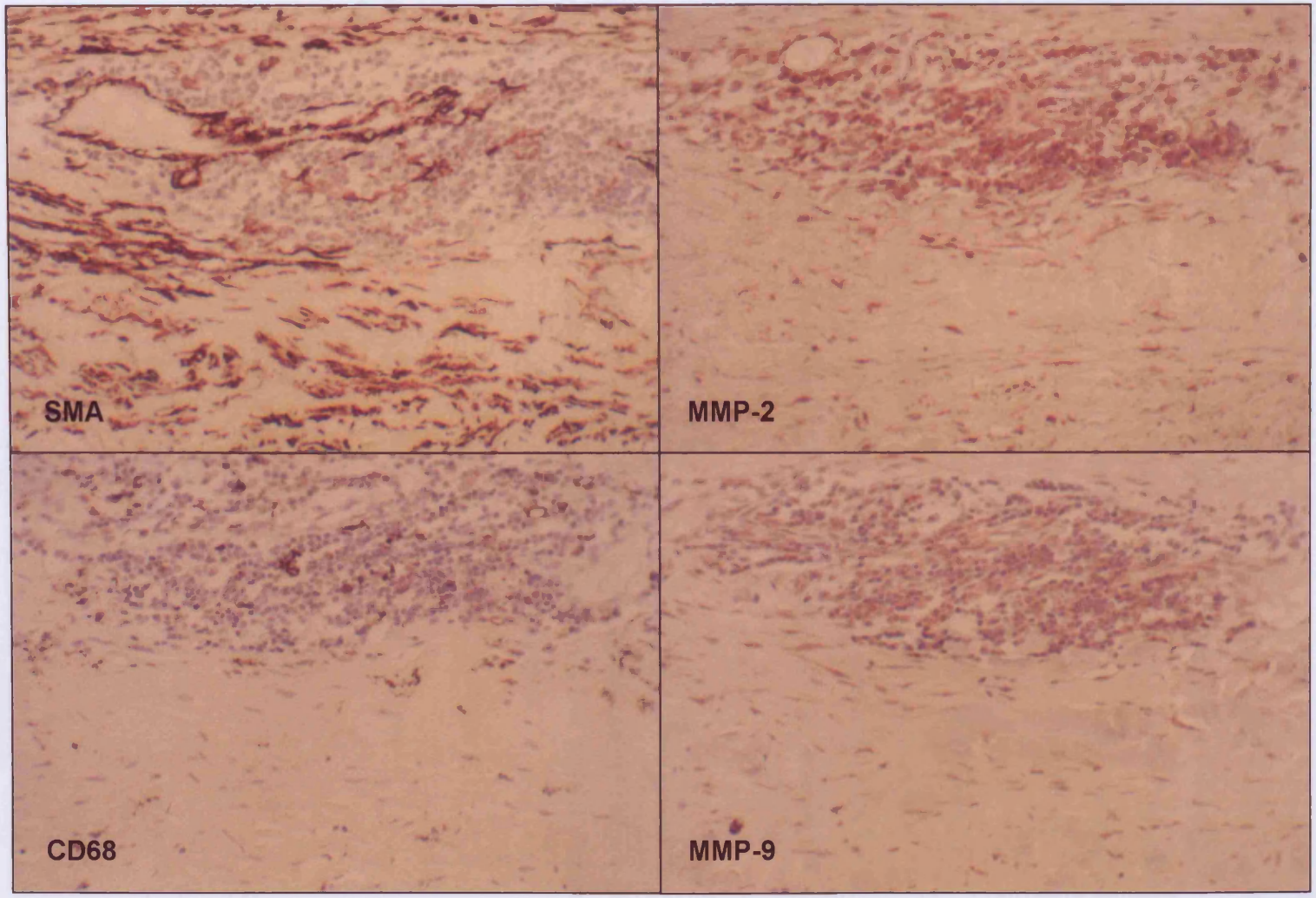


Figure 4.15 Immunohistochemical staining of aneurysmal aorta, with smooth muscle α actin (SMA), MMP-2, macrophage (CD68) and MMP-9 antibodies. This configuration demonstrated clear co-localisation of MMP-2 with SMCs and MMP-9 with macrophages.



4.4 *In Situ Hybridisation*

The primary focus of this thesis was to examine the role of the MMP-2/MT1-MMP/TIMP-2 enzyme-inhibitor system, and the potential for it to cause the genesis of AAAs by elastin damage. Previous investigators have used *in situ* hybridisation to examine closely the role of other elastolytic MMPs such as MMP-9,[195-197] and so this work was not repeated here. Briefly, these data showed that MMP-9 was predominantly expressed by inflammatory leukocytes within the wall of AAAs, as was MMP-1. As mentioned at the beginning of this chapter, whilst the production of MMP-2 has previously been addressed,[197] no studies to date have examined the production or expression of MT1-MMP in vascular tissue of any kind, a surprising omission given its role in MMP-2 activation. In the present study, the use of *in situ* hybridisation permitted a precise cellular source to be defined for MMP-2/MT1-MMP and TIMP-2 expression, in order to further clarify the involvement of this system in AAAs. Labelling of oligonucleotide probes was tested by dot blotting, as outlined in Section 3.5.2, and the results of these test strips provided the appropriate working concentration for the probes (Figure 4.16).

Figure 4.16 Test strips of the biotinylated oligonucleotide probes used in the *in situ* hybridisation experiments, indicating the sensitivity of the probes against salmon sperm DNA target, as indicated by an arrow. (a) MMP-2 probe, with sensitivity at 1pg/ml. (b) TIMP-2 probe, with sensitivity at 0.1pg/ml. (c) MT1-MMP probe, with sensitivity at 1pg/ml.

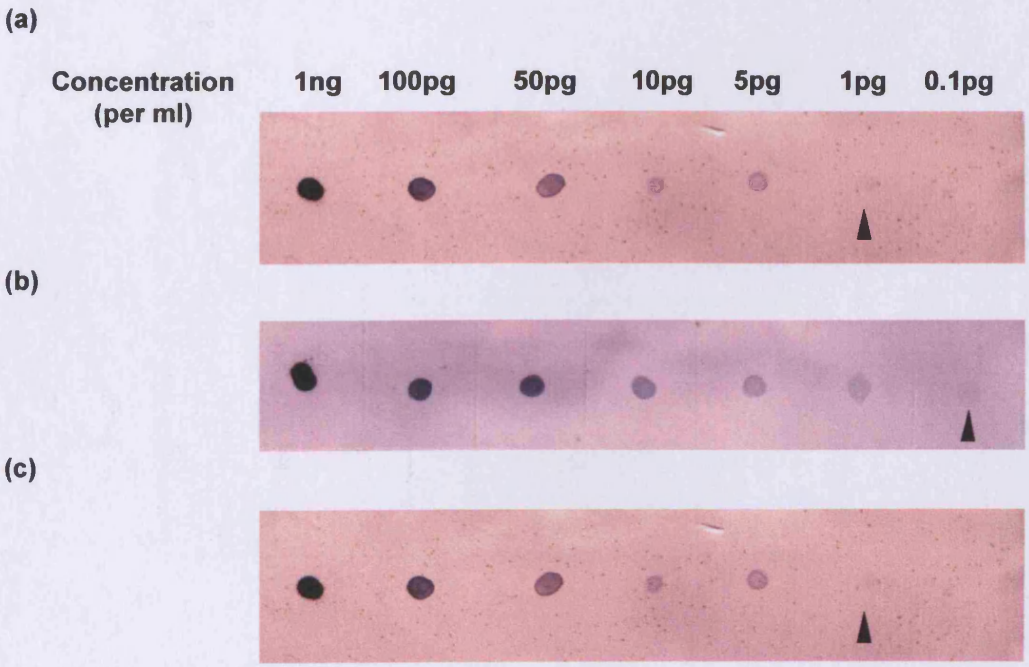


Figure 4.17 RNase H treated, negative control sections, demonstrating the lack of reactivity with the MMP-2 probe (x80). These sections were treated as usual, with the exception that they were incubated with RNase H for 1 hour at 37°C prior to probe hybridisation. No signal was obtained, indicating the lack of background reactivity produced by the detection steps of the protocol.

(a)

(b)

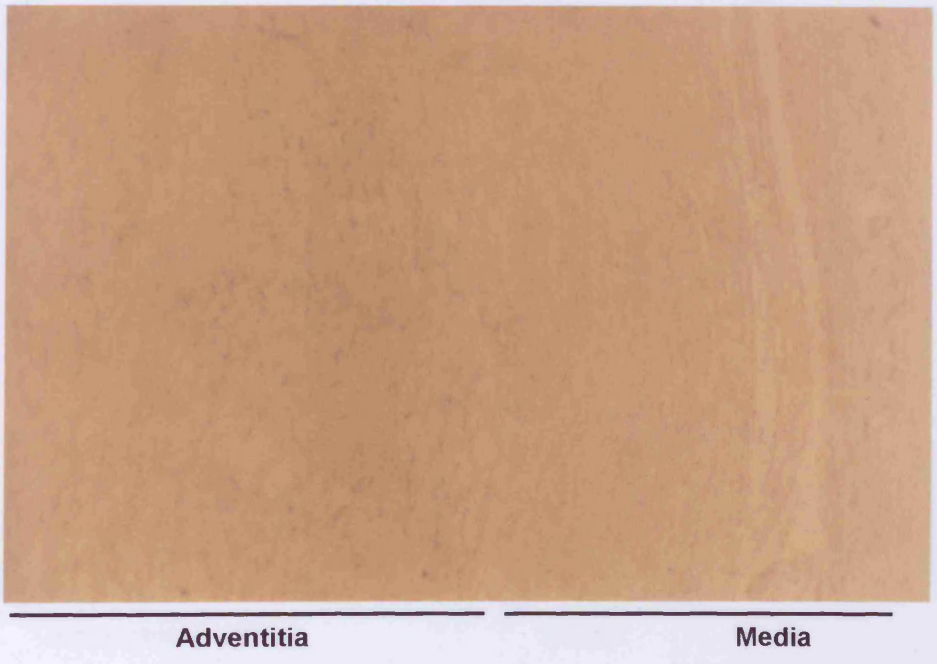


Figure 4.18 ***In situ*** **hybridisation of MMP-2 in (a) control aorta and (b) aneurysmal aorta (x80 and x320). Reactivity in control aorta matched that of immunohistochemical observations, localising to the medial SMCs, particularly on the adventitial side (left of figure). In AAAs, MMP-2 was also produced by SMCs, but also was detected in the inflammatory leukocytes present throughout the vessel wall.**

(a)

(b)

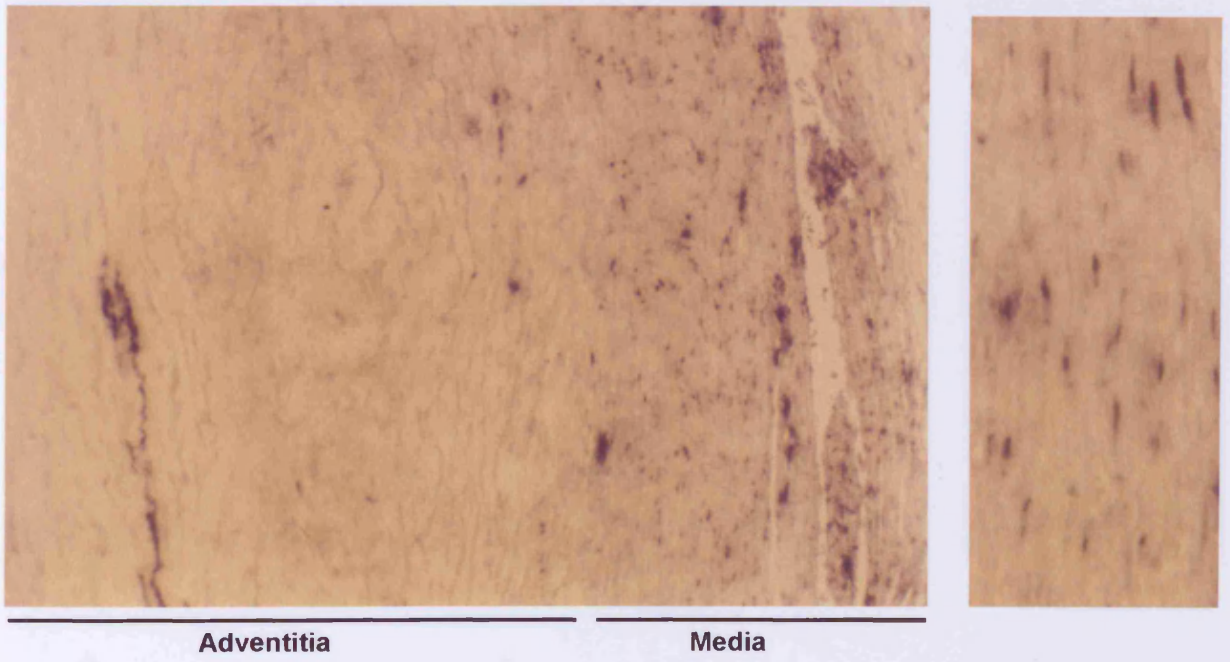


Figure 4.19 ***In situ*** **hybridisation of MT1-MMP in (a) control aorta and (b) aneurysmal aorta (x80 and x320). Like MMP-2, MT1-MMP was expressed by SMCs throughout the medial layer of control aorta. In AAAs the expression was detected in both SMCs and leukocytes in both the medial and adventitial layers.**

(a)

(b)

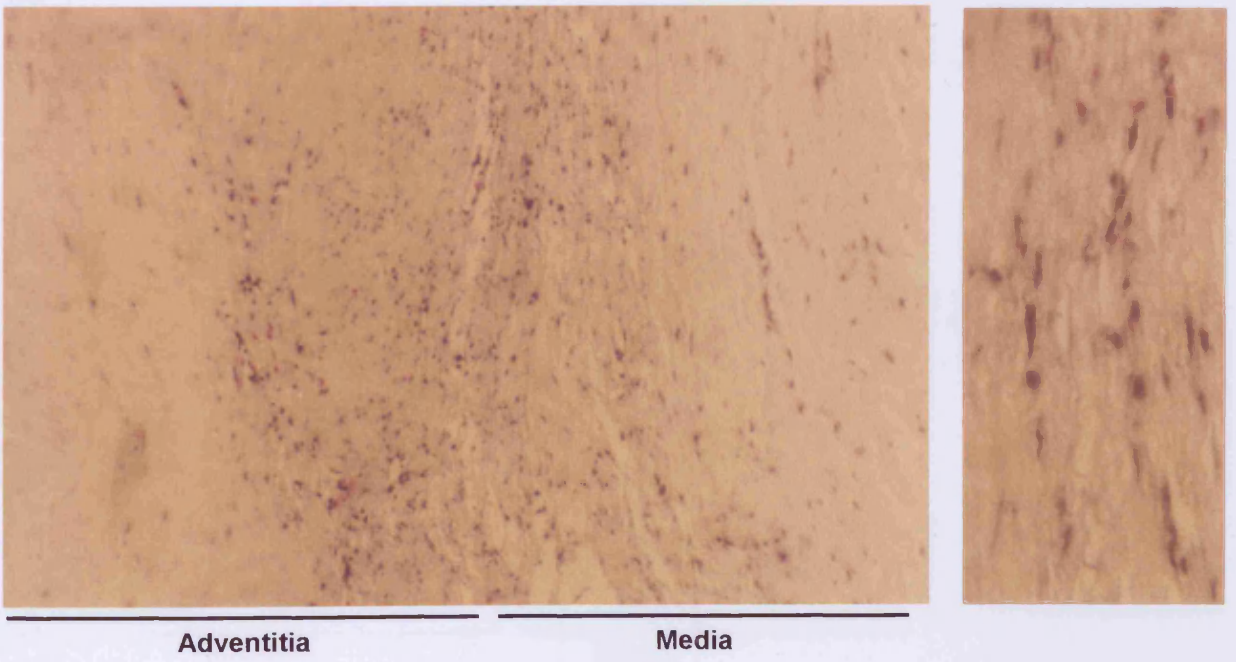
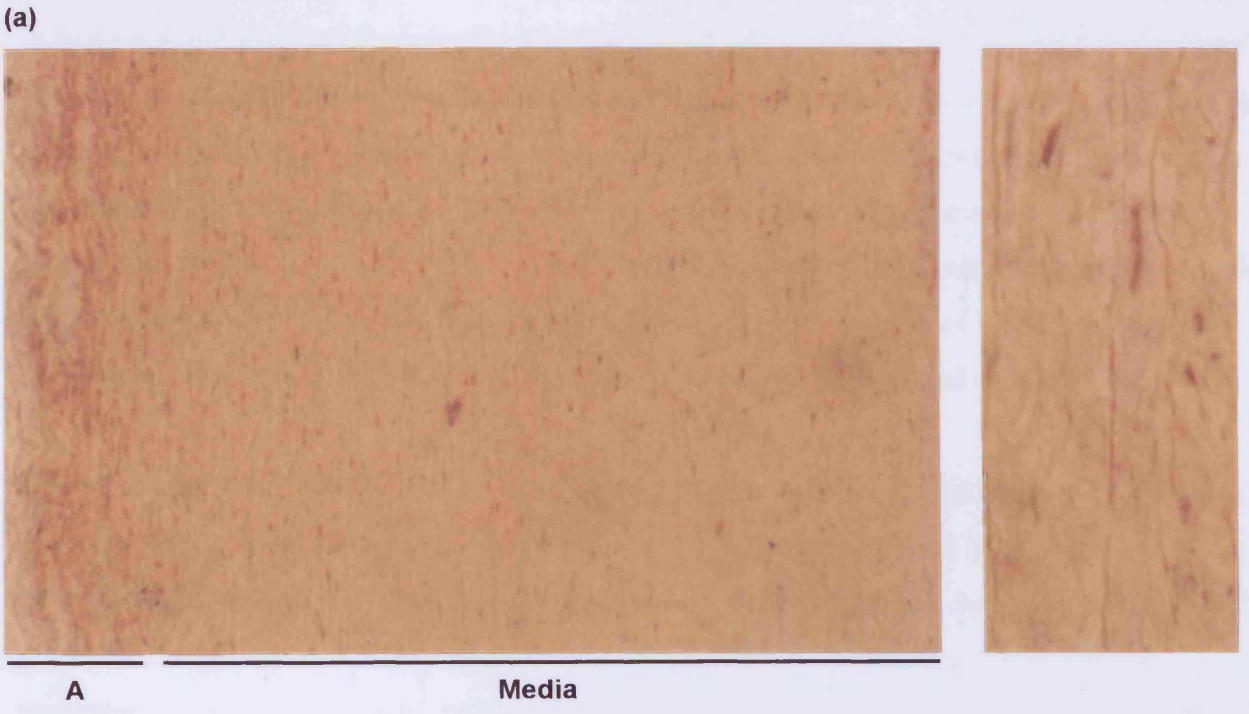


Figure 4.20 ***In situ*** **hybridisation of TIMP-2 in (a) control aorta and (b) aneurysmal aorta (x80 and x320). Control aorta exhibited TIMP-2 expression in the medial SMCs. AAAs contained widespread expression of TIMP-2, with cells throughout the medial and adventitial layers showing reactivity.**



4.5 *In Situ Zymography*

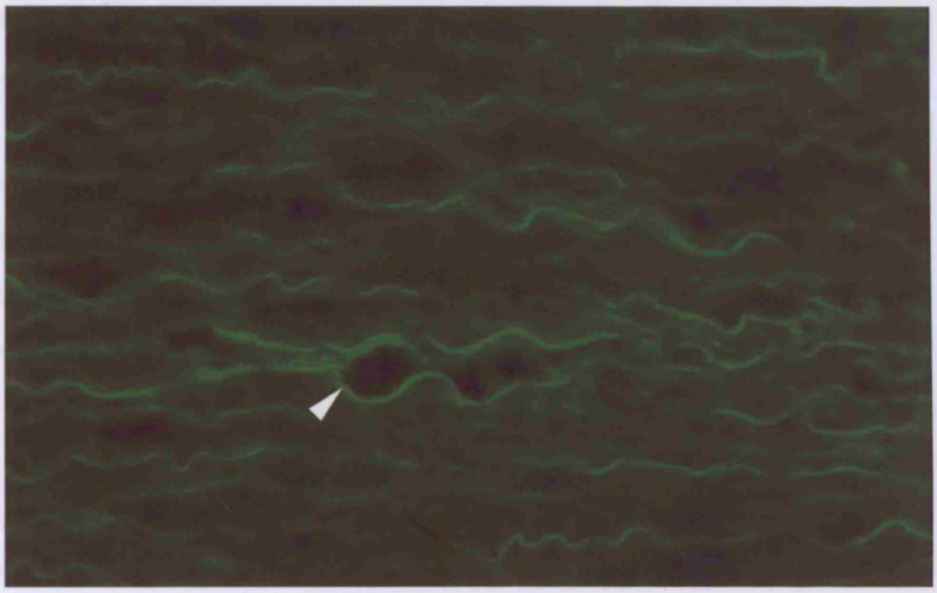
The use of *in situ* zymography represented a previously untried method of analysis of protease activity in the aortic wall. Very few instances of this technique exist within published literature, being confined to reports from Galis *et al*[333] and Mungall *et al.*[334] The former group reported the first instance of the technique in atheromatous tissue, in which MMP-3 and 10 activity was detected using resorufin-labelled casein as a substrate, and gelatinolytic activity detected using fluorosceinated gelatin or autoradiographic emulsion (containing gelatin). The technique presents an important opportunity to differentiate between latent and active forms of particular MMPs, information denied by the use of immunohistochemistry and *in situ* hybridisation. However, the major drawback is in its use of frozen tissue, which as previously mentioned is associated with a reduction in the microscopic integrity of tissue architecture. This is better preserved in formaldehyde-fixed, paraffin wax-embedded specimens.

The commercial availability of fluorosceinated gelatin suggested its use in the present study. The images produced revealed the site of gelatinolytic enzyme activity in the tissue sections of control and aneurysmal aorta. This permitted a distinction between latent and active MMP production. Figure 4.21 illustrates a typical result produced by sections of control and aneurysmal aorta. Control aorta exhibited diffuse gelatinolysis within the medial layer, which was particularly associated with the elastin fibres. This activity was abolished in the 1, 10, phenanthroline-treated sections, proving its identity as a metalloproteinase. Positive control slides revealed a moderate general increase in fluorescence, indicating that a small amount of latent MMP was present within the tissue. Again, this activity was mainly associated with the elastin fibres and also was present in the adventitial layer around the vasa vasorum.

The aneurysmal aortic tissue also contained gelatinolysis within the medial and adventitial layers, but the distribution of this reflected the disorganisation of the vessel wall architecture. Medial fluorescence was reduced to a thin band, co-localising with the elastin as illustrated earlier in the EVG sections. Adventitial fluorescence was limited, and associated with the inflammatory cells. This activity was also abolished by treatment with 1,10, phenanthroline. Positive control slides again indicated that a proportion of MMP within the aneurysm tissue was latent, its distribution matching that of the active enzyme.

Figure 4.21 ***In situ*** **zymography of (a) control aorta and (b) aneurysmal aorta (x320). Green fluorescence designated areas of active gelatinolysis. Strong regions of fluorescence are indicated with an arrow. Diffuse activity in the control aorta localised to elastin fibres, seen as wavy lines. This contrasted strongly with the AAA tissue, which exhibited highly localised regions of activity which co-localised with remaining elastin fibres, and other faint areas of activity within the media and adventitia.**

(a)



(b)

4.6 *Conclusions*

These data confirmed results presented in previous studies, demonstrating the production of a range of MMPs by resident aortic cells and inflammatory cells, particularly the importance of the inflammatory cells in the liberation of MMP-1 and 9, which are only seen in AAA tissue and not controls. The results also suggested that medial SMCs were a major source of MMP-2, MT1-MMP and TIMP-2 in aortic tissue. Furthermore these data suggested for the first time that the medial SMCs and the cells of the inflammatory infiltrate are expressing MT1-MMP. Thus, evidence is presented for the elastolytic capacity of the environment surrounding the SMCs and associated elastin in the arterial media.

Also shown for the first time was the MMP activity within the control and aneurysmal aortic wall, as visualised by *in situ* zymography. The identification and localisation of MMPs and TIMPs by specific antibody or probe binding was augmented by demonstration of active enzyme associated with the elastin fibres. By interpolation from immunohistochemical analysis, this activity was most likely to represent active MMP-2, both in control and aneurysmal aortic tissue, as no other gelatinolytic MMP was consistently localised to the SMCs of the medial layer. Thus, their secreted MMP-2 may be activated by MT1-MMP in complex with TIMP-2, causing fragmentation of elastin and the initiation of a proteolytic/inflammatory reaction, leading to aneurysmal degeneration.

An important point to note from this chapter was the lack of leukocytes in the medial layer of control aortic tissue. The absence of inflammatory cells from these specimens suggested that their presence in AAAs was most likely as a secondary phenomenon to an initial trauma. There was little evidence to support the view that leukocytes may be responsible for such an event and this may strengthen the argument that the resident SMCs were the most likely source of proteases causing damage to the vessel wall in the first instance.

Histological analyses such as these permitted detailed observations to be made on the identity, source and activity status of the individual MMPs and TIMPs in control and AAA tissue. However, no quantitative data was obtained, primarily because the morphology of the diseased tissue was very different from normal artery, precluding direct stereological comparisons. Furthermore, no aetiological observations were possible, as the use of end-stage aneurysmal tissue precluded the separation of pathogenetic factors from secondary

degenerative changes, such as inflammation and loss of medial SMCs. To quantitatively establish the expression of MMPs and TIMPs, further studies on isolated cells were necessary.

CHAPTER FIVE

Results:

Smooth Muscle Cells

5.1 *Introduction*

Previous research took a broad approach to the analysis of proteases in the aneurysm wall by examining homogenates of whole tissue. By taking this approach, the influence of all cell types present within the aneurysmal aortic wall were inseparable. Thus, meaningful conclusions on the role of resident mesenchymal cells, or newly introduced inflammatory cells, were impossible to define. The approach of this thesis was to examine more closely the role of individual cell types, particularly the medial SMCs, and their contribution to the collection of events which constitute the developing aneurysm.

The demonstration of increased elastolysis as an early event in AAA development, combined with the knowledge that SMCs are the major resident cell type present in control and aneurysmal aortic tissue, led to the speculation that SMCs may be responsible for the production of proteolytic enzymes which cause elastin destruction. In order to study these cells in detail it was first necessary to isolate and grow them *in vitro*, and so aortic tissue from control patients and those with AAAs was obtained for this purpose. Once established, culture medium samples and RNA were extracted from the cells. This permitted comparisons of the MMP and TIMP production and expression of the cell lines to be made, and the identification of any primary differences between those cells which may influence ECM metabolism in the aortic wall.

Also tested in this chapter was the concept that vascular cells in patients predisposed to aneurysm formation may respond to circulating components in the blood. Serum samples were collected from patients with atherosclerosis, AAAs or no vascular disease, and used to culture saphenous vein SMCs derived from control patients. The influence of this serum on the MMP expression of these cells was then tested to assess the presence of such blood-borne, circulating components.

Summary Of The Aims Of This Chapter

- To establish SMC lines from control and aneurysmal aortic wall for the purposes of comparing their proliferation rate, and MMP/TIMP production
- To confirm the identity of these cells by immunofluorescent detection of cell-specific markers
- To compare MMP and TIMP production levels of the cell lines by gelatin zymography, ELISA and western blotting
- To confirm the gene expression of the MMP-2/MT1-MMP/TIMP-2 genes by RT-PCR
- To compare the expression levels of the MMP-2/MT1-MMP/TIMP-2 genes by northern blotting

5.2 *SMC Culture*

Establishment of aortic SMC lines was difficult. Procurement of age-matched control aorta was rare through normal operating procedures or organ donors, and eventually cadaveric tissue was used, which in all cases was non-viable. Of 51 control aortic samples obtained, only 6 cell lines were established, all from aorto-bifemoral grafting procedures. Tissue was taken from the aortic wall 2cm distal to the left renal vein, as previously described. Aneurysmal aorta was readily available, and SMCs were relatively easy to culture. 8 SMC lines from patients with AAAs were established to provide a similar number of cases in each group. The demographics of the patients from whom tissue was taken are described in Table 5.1.

Analysis of the patient groups suggested that the median ages between controls and patients with AAAs were not significantly different (control median age 68.5 (61.22-75.11), AAA median age 74.5 (69.42-78.08); n=14, U=11.00, p=0.1079), as established by Mann-Whitney analysis. All of the patients were non-smokers or ex-smokers, but the validity of these claims was not tested by serum cotinine assays.

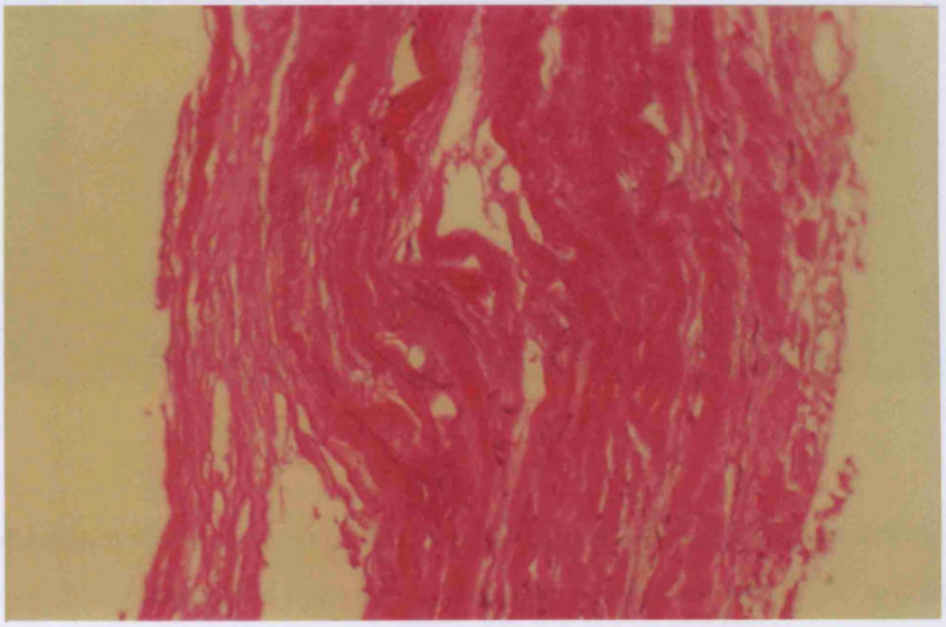
Table 5.1 **List of patients from whom aortic tissue was obtained for SMC studies.**

Patient	Age	Sex	Aortic Diameter (cm)
Control 1	58	M	<2.5
Control 2	67	M	<2.5
Control 3	75	F	<2.5
Control 4	75	M	<2.5
Control 5	70	M	<2.5
Control 6	64	F	<2.5
AAA 1	81	M	7.7
AAA 2	72	M	6.0
AAA 3	67	M	6.0
AAA 4	78	M	7.0
AAA 5	77	M	9.0
AAA 6	77	M	5.5
AAA 7	70	M	7.0
AAA 8	68	M	6.5

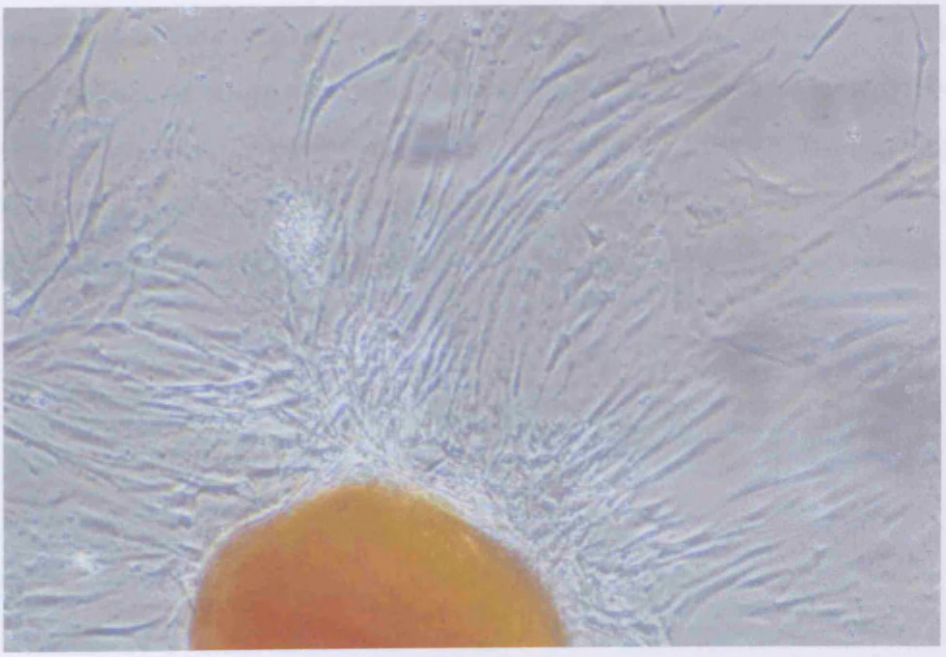
Figure 5.1a illustrates a haemotoxylin and eosin (H&E) stained, transverse section of the aortic medial layer, dissected from aneurysmal tissue, showing the nuclei of the SMCs which were subsequently cultured. This section was primarily used to ensure the absence of intimal and adventitial tissue from the dissection process, which may have led to contamination of subsequent SMC cultures with fibroblasts and/or endothelial cells. Figure 5.1b shows the outgrowth of SMCs from an explant of medial tissue as described in Section 3.1.1. Cells were seen to proliferate radially in a striated pattern, and formed a typical hill-and-valley morphology common to SMCs in culture.[335] Positive identification of the cells as smooth muscle was achieved by immunofluorescent detection of α-actin filaments, as illustrated in Figure 5.1c.

Figure 5.1 **(a) Haemotoxylin and eosin stained section of medial layer of human aneurysmal aorta, illustrating the nuclei of interspersed SMCs, and confirming this tissue as the medial layer of the vessel (x160). (b) Outgrowth of SMCs from a medial explant of aortic tissue *in vitro*, illustrating the characteristic striated pattern of SMC growth (x100). (c) Immuno-fluorescent detection of smooth muscle α-actin filaments in cultured cells (x320).**

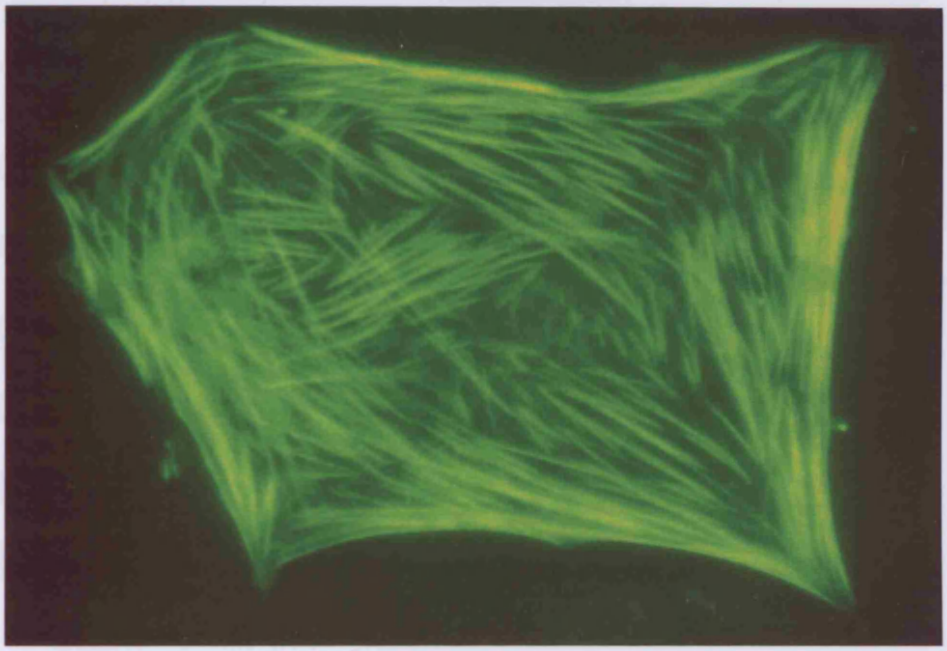
(a)



(b)



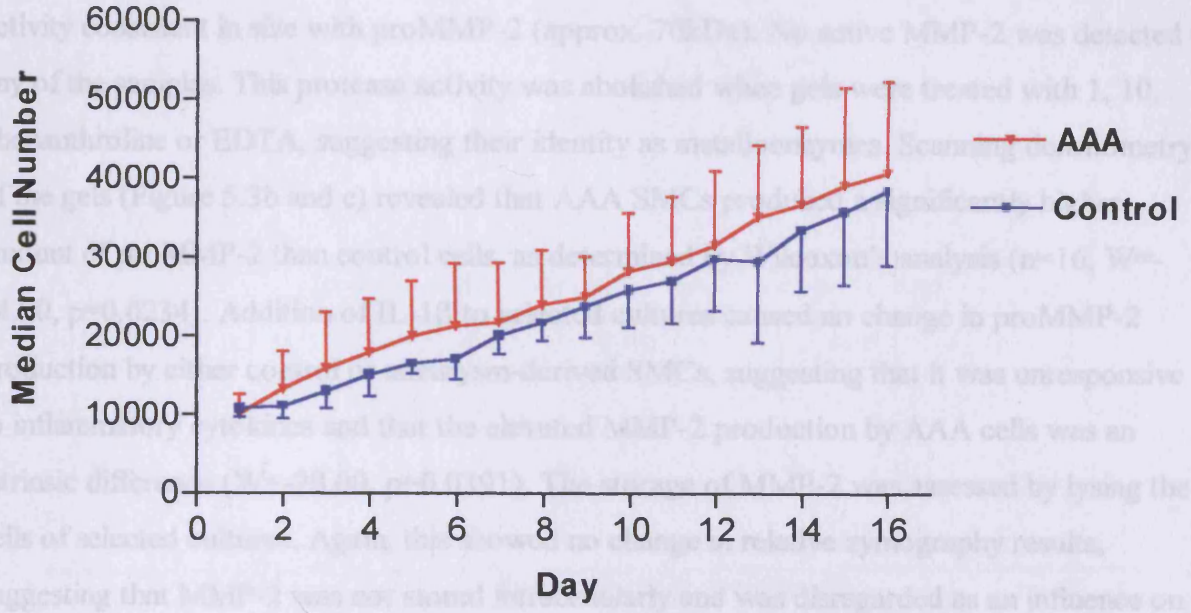
(c)



Proliferation rates of the control and aneurysm cell lines were established as described in Chapter Three. Proliferation has been associated with MMP expression[336,337] and this was deemed an important factor when assessing gene expression of the cells. It may also reflect primary differences between the two patient groups. Figure 5.2 demonstrates that there was no significant difference in the proliferation rate between AAA and control cells. This was assessed by calculating the areas under the curve of each day for each cell line, and comparing the two groups with a Mann-Whitney test ($n=12$, $U=15.00$, $p=0.6991$). This finding suggested that any differences detected in MMP expression were more likely to reflect primary changes in the regulation of gene transcription and were not a function of increased mitotic activity.

Figure 5.2 (a) Median cell numbers with 95% confidence intervals of control and aneurysmal SMCs over 16 days, illustrating their similar rates of proliferation. (b) Summary of proliferation data, expressed as median areas with 95% confidence intervals and p values.

(a)



(b)

Median area under curve with 95% confidence intervals ($x10^5$)		
Control	**AAA**	**p Value**
3.74 (2.71-4.99)	3.17 (2.84-4.06)	0.6991

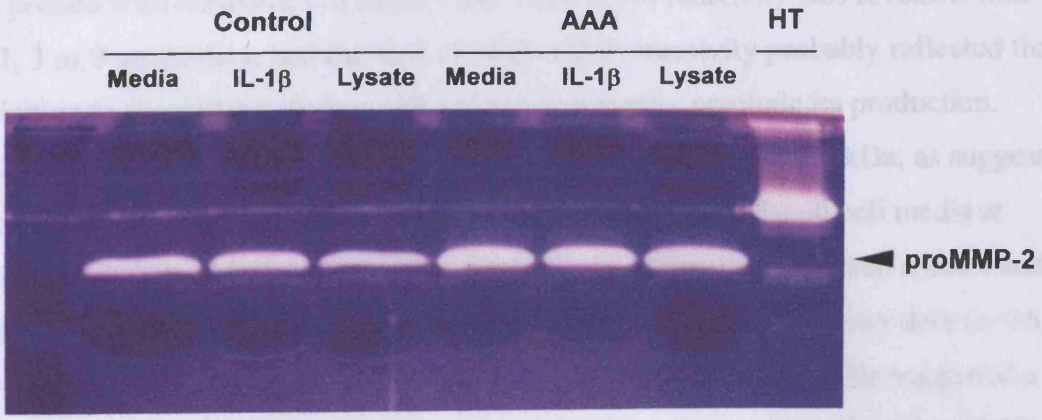
5.3 *MMP And TIMP Production By Smooth Muscle Cells*

5.3.1 *Gelatin Zymography Analysis Of SMC Culture Media Samples*

Conditioned serum-free medium from $1x10^5$ cells was collected from all cell lines and used in zymographic, western and ELISA analyses. Figure 5.3a shows a typical gelatin zymogram comparing medium from control cells and AAA cells, all of which contained proteolytic activity consistent in size with proMMP-2 (approx. 70kDa). No active MMP-2 was detected in any of the samples. This protease activity was abolished when gels were treated with 1, 10, phenanthroline or EDTA, suggesting their identity as metalloenzymes. Scanning densitometry of the gels (Figure 5.3b and c) revealed that AAA SMCs produced a significantly higher amount of proMMP-2 than control cells, as determined by Wilcoxon's analysis (n=16, W=-34.00, p=0.0234). Addition of IL-1β to selected cultures caused no change in proMMP-2 production by either control or aneurysm-derived SMCs, suggesting that it was unresponsive to inflammatory cytokines and that the elevated MMP-2 production by AAA cells was an intrinsic difference (W=-28.00, p=0.0391). The storage of MMP-2 was assessed by lysing the cells of selected cultures. Again, this showed no change in relative zymography results, suggesting that MMP-2 was not stored intracellularly and was disregarded as an influence on the data presented here (W=-28.00, p=0.0234).

Figure 5.3 (a) Gelatin zymogram showing elevated proMMP-2 production by aneurysmal smooth muscle cells compared to control cells. (b) Scattergram of densitometry readings, with median and p value indicated. (c) Summary of scanning densitometry data from gelatin zymography of smooth muscle cell media, expressed as median optical density in arbitrary units with 95% confidence intervals.

(a)



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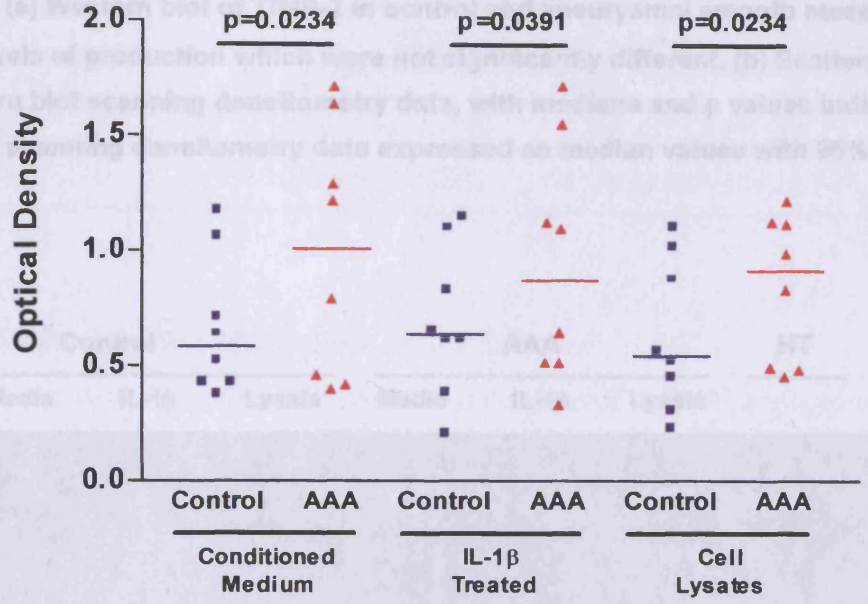


(c)

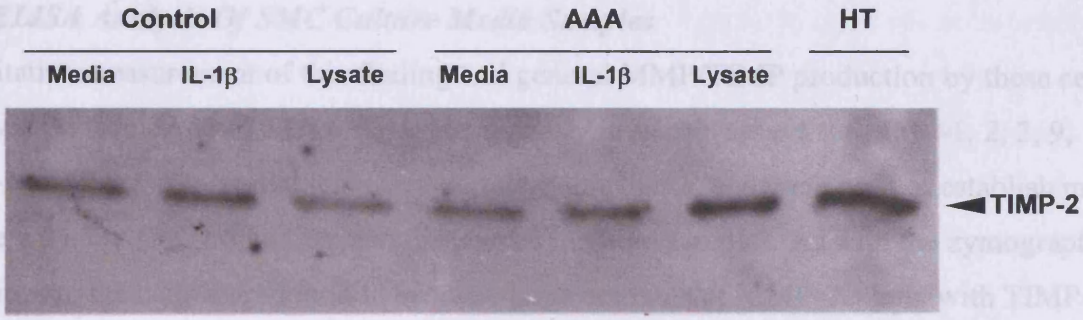
	Median optical density with 95% confidence intervals		
	Control	AAA	p Value
Conditioned Medium	0.587 (0.422-0.924)	1.008 (0.539-1.451)	0.0234
IL-1β Treated	0.639 (0.428-0.970)	0.873 (0.509-1.370)	0.0391
Cell Lysates	0.547 (0.365-0.913)	0.915 (0.575-1.112)	0.0234

5.3.2 *Western Blotting Analysis Of SMC Culture Media Samples*

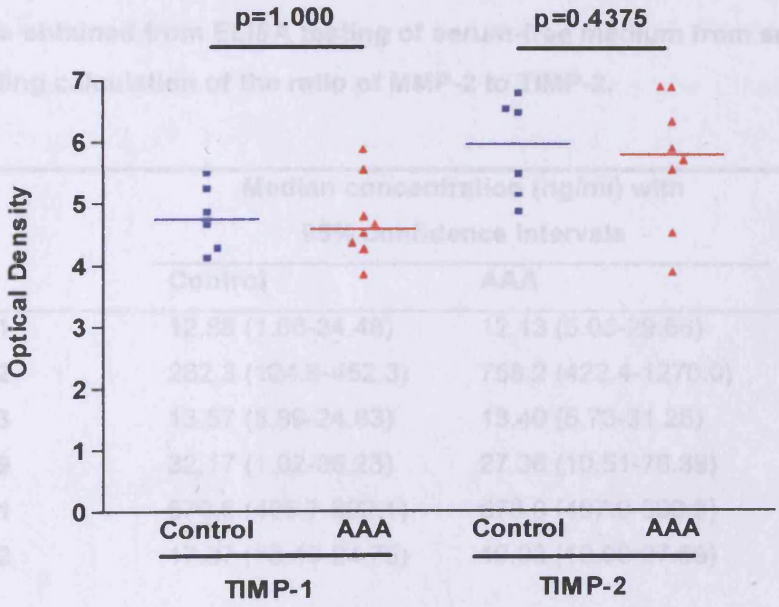
Further analysis of MMP/TIMP production was undertaken by western blotting. All cell line media were probed with antibodies to MMPs and TIMPs. No reactivity was revealed with anti-MMP-1, 3 or 9 antibodies, and the lack of MT1-MMP reactivity probably reflected that enzyme's binding to the cell membrane and did not necessarily preclude its production. However, all cultures contained anti-MMP-2 reactive material at around 70kDa, as suggested by the zymography data. TIMP-1 and TIMP-2 reactivity was strong for all cell media at around 30kDa and 20kDa respectively, with no significant differences between control and AAA samples, as determined by Wilcoxon's analysis of scanning densitometry data (n=16, TIMP-1 W=-1.000, p=1.000; TIMP-2, W=9.000, p=0.4375) (Figure 5.4). This suggested a potential imbalance between MMP-2 and TIMP-2 production may exist in the AAA SMCs.

Figure 5.4 **(a) Western blot of TIMP-2 in control and aneurysmal smooth muscle cell media, revealing levels of production which were not significantly different. (b) Scattergram of TIMP-1 and 2 western blot scanning densitometry data, with medians and p values indicated. (c) Summary of scanning densitometry data expressed as median values with 95% confidence intervals.**

(a)



(b)



(c)

	Median optical density with 95% confidence intervals		
	Control	**AAA**	**p Value**
TIMP-1	4.748 (4.214-5.323)	4.593 (4.173-5.298)	1.000
TIMP-2	5.596 (5.002-6.724)	5.788 (4.808-6.592)	0.4375

5.3.3 *ELISA Analysis Of SMC Culture Media Samples*

Quantitative measurement of this finding and general MMP/TIMP production by these cell lines was performed by ELISA tests. Commercially available assays for MMP-1, 2, 3, 9, TIMP-1 and 2 became available midway through this thesis, and were used to establish more precisely the level of production into the culture medium samples. As with the zymography experiments, the cells were found to produce large amounts of MMP-2, along with TIMP-1. No significant amounts of MMP-1, 3 or 9 were detected, and the data for TIMP-2 were surprisingly low. ELISA testing for MT1-MMP was not possible due to the membrane-bound nature of this enzyme. Table 5.2 shows the data obtained from these assays.

Table 5.2 Data obtained from ELISA testing of serum-free medium from smooth muscle cell cultures, including calculation of the ratio of MMP-2 to TIMP-2.

	Median concentration (ng/ml) with 95% confidence intervals		
	Control	**AAA**	**p value**
MMP-1	12.88 (1.86-34.48)	12.13 (5.05-29.66)	0.8518
MMP-2	262.3 (124.9-452.3)	758.2 (422.4-1270.0)	0.0080
MMP-3	13.57 (5.89-24.83)	13.40 (5.73-31.25)	0.9497
MMP-9	32.17 (1.02-86.23)	27.36 (10.51-76.39)	0.7546
TIMP-1	570.8 (405.7-892.1)	678.0 (497.9-999.9)	0.6620
TIMP-2	17.37 (13.49-24.75)	19.93 (10.98-27.59)	1.0000
MMP-2: TIMP-2 Ratio	14.35 (6.66-23.63)	38.34 (14.52-108.60)	0.0080

The suspicion of an imbalance between MMP-2 and TIMP-2 production by SMCs in AAAs, as suggested by the western blotting data, was strengthened by the calculation of the ratio

between MMP-2 and TIMP-2 in control and AAA media samples (Table 5.2). This revealed that the ratio in AAA cells was twice that in controls, and Mann Whitney analysis of these ratios revealed that this difference was significant (n=14, U=4.00 , p=0.0080).

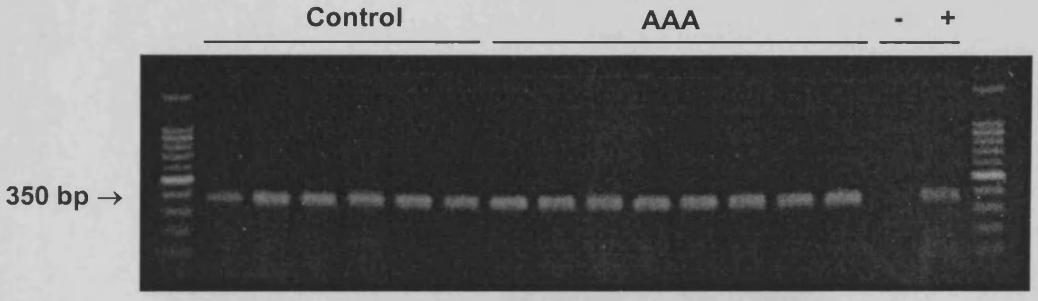
5.4 *MMP And TIMP Gene Expression By Smooth Muscle Cells*

Gene expression of the MMPs/TIMPs was confirmed by RT-PCR, using specific primers as described in Chapter Three. Figure 5.5 illustrates the products of typical PCR reactions and demonstrates that MMP-2, MT1-MMP and TIMP-2 were expressed by both control and AAA SMCs. To quantify any differences at the level of gene expression, northern analysis was employed. Equal amounts of RNA from control and AAA SMCs was loaded and probed with MMP-2, MT1-MMP and TIMP-2 oligonucleotide cocktails. A GAPDH probe was used to confirm equal loading of RNA between samples. The results obtained further supported those observed with zymography and ELISA analysis of culture media samples.

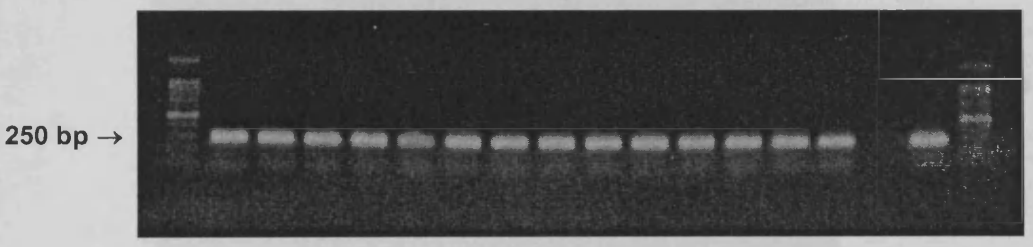
MMP-2 mRNA was significantly increased in AAA samples compared to controls, as determined by Wilcoxon's analysis of scanning densitometry data (n=16, W=-21.00, p=0.0313), and MT1-MMP and TIMP-2 mRNA were not significantly different (MT1-MMP, W=-3.000, p=0.8438; TIMP-2, W=-3.000, p=0.8438) (Figure 5.6). Analysis was also carried out on the ratio between the data for MMP-2 and GAPDH. This also illustrated a significantly elevated expression of MMP-2 by the aneurysm-derived SMCs (n=14, W=8.00, p=0.0426).

Figure 5.5 1% Agarose gels of RT-PCR products from control and aneurysmal smooth muscle cell RNA. (a) MMP-2 (b) TIMP-2 (c) MT1-MMP (d) GAPDH. All gels contained 100bp markers, control and AAA reaction products as indicated, plus no *Taq* control (-) and HT1080 RNA control (+).

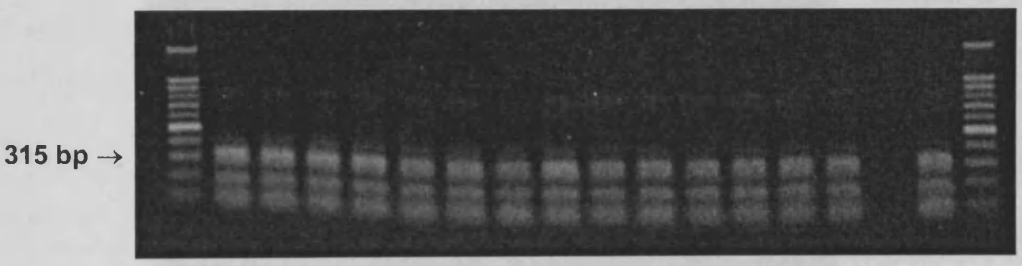
(a)



(b)



(c)



(d)

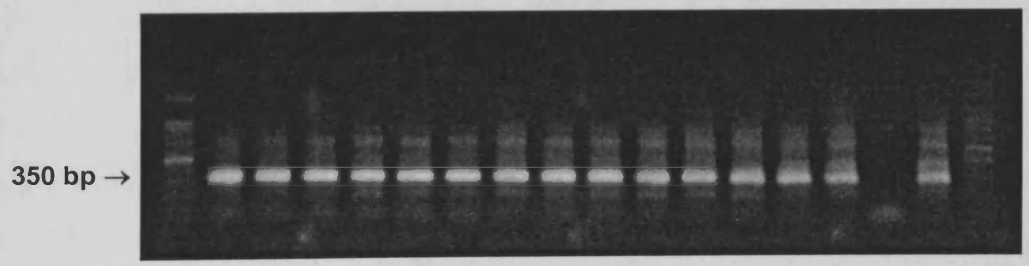
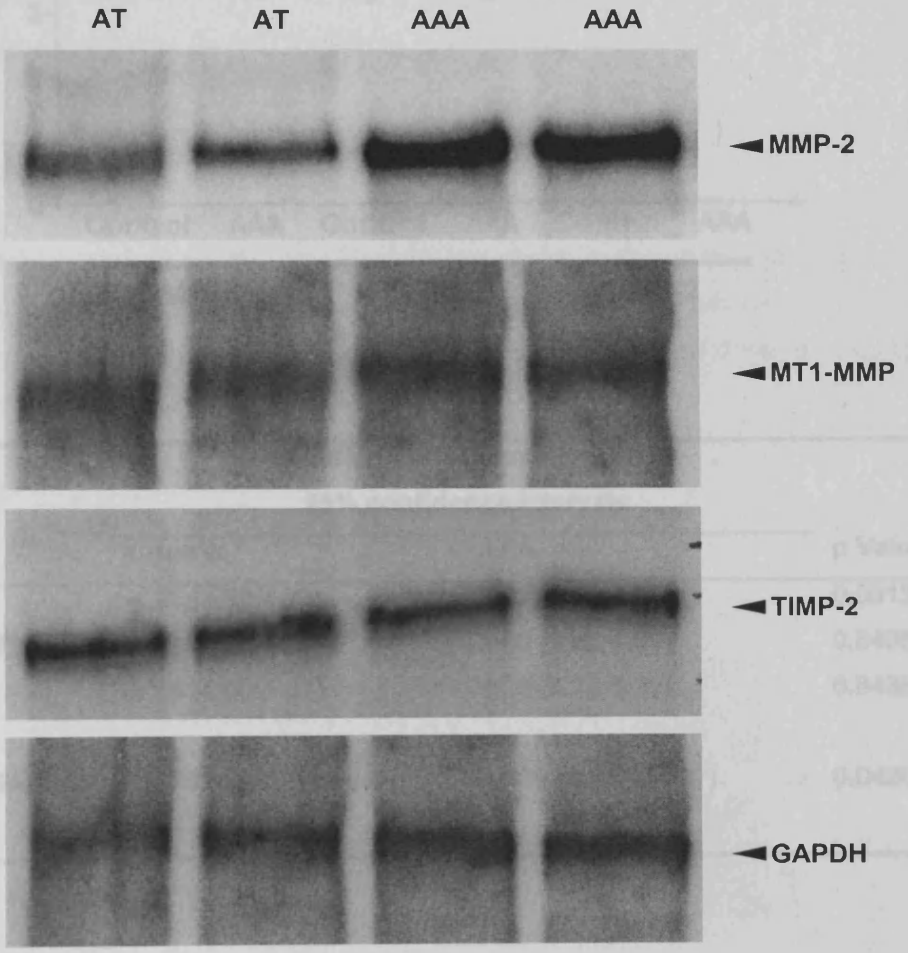
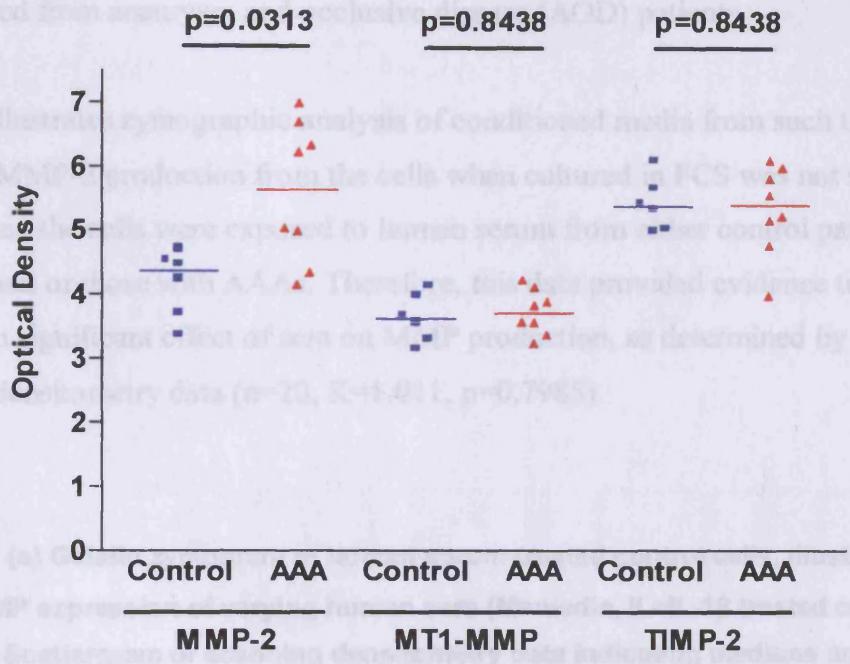


Figure 5.6 **(a) Northern blot of 5μg control and aneurysmal smooth muscle cell RNA probed with either MMP-2, MT1-MMP, TIMP-2 or GAPDH as indicated. This illustrates the elevated level of MMP-2 RNA in aneurysmal samples, and the similar levels of MT1-MMP and TIMP-2 transcript. (b) Scattergram of scanning densitometry data, indicating medians. (c) Summary of scanning densitometry data expressed as median values with 95% confidence intervals.**

(a)



(b)



(c)

	Median optical density with 95% confidence intervals		
	Control	AAA	p Value
MMP-2	4.37 (3.62-4.73)	5.63 (4.67-6.52)	0.0313
MT1-MMP	3.62 (3.24-4.03)	3.69 (3.42-4.01)	0.8438
TIMP-2	5.38 (4.99-5.87)	5.39 (4.73-5.92)	0.8438
MMP-2:GAPDH Ratio	1.176 (0.965-1.356)	1.559 (1.246-1.787)	0.0426

5.5 *Serum Influence On MMP Production*

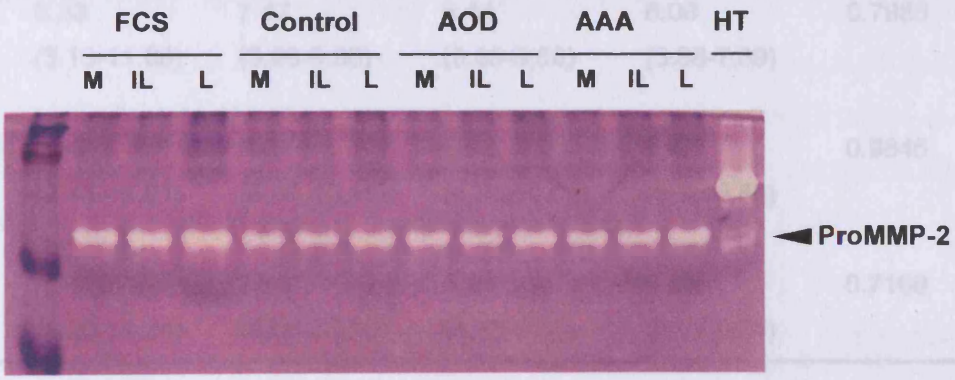
It was speculated that the differences reported here were the product of a genetic predisposition to increased MMP-2 production in the aneurysm cells. However, it is possible that the cells may have undergone a temporary phenotypic change *in vivo*, perhaps as a result of prolonged exposure to inflammatory conditions. As the cell lines were passaged two or three times, such changes were unlikely to have remained *in vitro*. However, to attempt to mimic the stimuli experienced by the cells *in vivo*, and thus test their responsiveness to a

blood-borne agent *in vitro*, saphenous vein SMCs were cultured in the presence of human serum derived from aneurysm and occlusive disease (AOD) patients.

Figure 5.7 illustrates zymographic analysis of conditioned media from such treated cells. The level of proMMP-2 production from the cells when cultured in FCS was not significantly changed when the cells were exposed to human serum from either control patients, those with atherosclerosis or those with AAAs. Therefore, this data provided evidence to suggest that there was no significant effect of sera on MMP production, as determined by Kruskall-Wallis analysis of densitometry data (n=20, K=1.011, p=0.7985).

Figure 5.7 (a) Gelatin zymogram of human serum-treated control cells, illustrating the lack of effect on MMP expression of varying human sera (M=media, IL=IL-1β treated cells, L=cell lysates). (b) Scattergram of scanning densitometry data indicating medians and p values. (c) Summary of scanning densitometry data expressed as median optical density readings with 95% confidence intervals.

(a)



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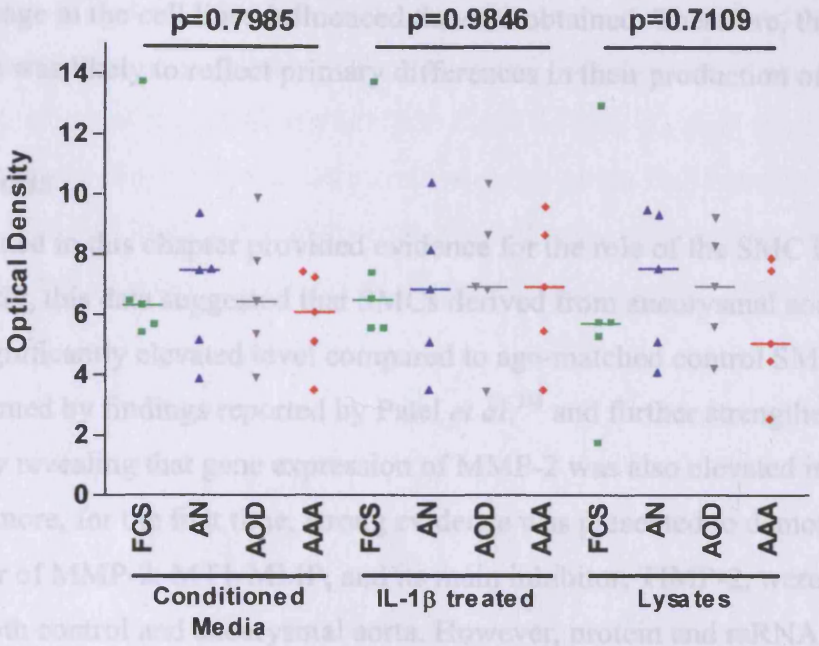


(c)

	Median optical density with 95% confidence intervals				
	FCS	Control	AOD	AAA	p Value
Conditioned Medium	6.33 (3.13-11.89)	7.47 (3.99-9.36)	6.44 (3.83-9.50)	6.08 (3.86-7.89)	0.7985
IL-1β Treated	6.49 (3.45-12.01)	6.83 (3.47-10.11)	6.92 (4.05-10.03)	6.92 (3.78-9.86)	0.9846
Cell Lysates	5.71 (1.20-11.36)	7.54 (4.08-10.15)	6.94 (4.35-9.36)	5.05 (2.72-8.24)	0.7109

These data are open to the criticism that as the sera used in the experiments were not heat treated, the complement proteins remaining within the samples may influence MMP production by the cultured cells. However, heat treatment would also have destroy any factors contained within the sera which these experiments aimed to detect. It was considered reasonable therefore, not to treat the sera, in order that any differences may be revealed. Whilst no firm conclusions could be drawn from these experiments, they suggested that patient serum did not appear to modulate SMC MMP production directly. Furthermore, the

use of SMCs after two or three passages in culture meant that it was unlikely that a temporary phenotypic change in the cell lines influenced the data obtained. Therefore, the data collected from the SMCs was likely to reflect primary differences in their production of MMPs.

5.6 *Conclusions*

The data presented in this chapter provided evidence for the role of the SMC in AAA disease. Most importantly, this data suggested that SMCs derived from aneurysmal aorta produce MMP-2 at a significantly elevated level compared to age-matched control SMCs. The present data was confirmed by findings reported by Patel *et al*,[338] and further strengthened this line of investigation by revealing that gene expression of MMP-2 was also elevated in aneurysmal SMCs. Furthermore, for the first time, strong evidence was presented to demonstrate that the *in vivo* activator of MMP-2, MT1-MMP, and its main inhibitor, TIMP-2, were produced by the SMCs of both control and aneurysmal aorta. However, protein and mRNA levels of both were not significantly different.

The main implication of these findings is that a potential imbalance between MMP-2 and TIMP-2 may have occurred *in vivo* in aneurysm patients, potentially causing increased net proteolysis. This suggestion was augmented by comparison of the MMP-2:TIMP-2 ratio in control and AAA SMCs, which was calculated to be more than double in the latter. Such a situation may have produced the degeneration of elastin characteristic of early aneurysms and act as the initiating event in a cascade of ECM catabolism. By extension from the histological studies, it was strongly suggested that the three components of the MMP-2/MT1-MMP/TIMP-2 system were present in the aortic medial layer and that these components were expressed by the resident SMCs. As the probable site of elastolytic initiation of the aneurysm, these findings were regarded as highly important.

The absence of any modulation of MMP production or activation in response to serum derived from AAA or AOD patients strongly suggests that blood-borne factors are not important in the level of SMC MMP production. It is more likely therefore that the AAA SMCs are inherently predisposed to increased MMP-2 expression.

Thus, the data in this chapter presented evidence to support the theory that medial SMCs may be responsible for the increased production of elastolytic MMP-2 that brings about destruction

of aortic wall structural elements in abdominal aneurysms. It further suggests that the accessory proteins MT1-MMP and TIMP-2, which are necessary for the activity of MMP-2, are also produced by the medial SMCs. This data strengthens the central notion of this thesis, that the SMCs produce all elements of the MMP-2/MT1-MMP/TIMP-2 system that are necessary for matrix destruction, and therefore may bring about the degenerative changes characteristic of AAAs. To progress this theory, the following chapter examined the ability of MMP-2 to participate in genesis of aneurysm-like changes in a model of disease.

CHAPTER SIX

Results:

The Porcine Aortic Model Of AAA

And The Effect Of EDP's

On MMP-2 Production

6.1 *Introduction*

Data from the previous chapter, combined with the studies of Freestone *et al*[185] has presented compelling evidence that a generalised tendency to vascular dilatation and the formation of aneurysms may be a result of increased production of elastolytic MMP-2 by vessel wall SMCs. Therefore, MMP-2 may be considered a fundamental factor in the initiation of aneurysmal disease. To test this supposition, a model of aneurysm formation based on organ culture of porcine aorta[325] was modified to examine the direct effects of MMP-2 enzyme on the wall of normal aorta.

The Wills model of AAAs was developed to illustrate the ability of the resident cells of the aortic wall to mediate a proteolytic reaction following an initial trauma, and thereby bring about degeneration of the medial ECM in a manner characteristic of aneurysms. The initial trauma was provided by temporary exposure of the porcine aorta to pancreatic elastase (a serine protease), which was washed away after 24 hours, followed by a 14 day culture period. During this period, induction of MMP production by resident SMCs was revealed, events which the authors speculate was a response to the liberation of elastin derived peptides from damaged elastin. The MMPs produced were detected by zymography of tissue homogenates, and this revealed that the elastase treated tissue contained elevated MMP-2 and MMP-9 compared to untreated controls, which caused destruction of much of the arterial elastin. Thus, the initial elastase treatment, which could not have brought about the profound loss of elastin, induced a proteolytic cascade, mediated by the resident SMCs, which resulted in severe ECM degeneration mirroring that of AAAs. Further developments of this model have proved that the elastolysis is mediated by MMPs and not the initial elastase, by using MMP inhibitor compounds such as Doxycycline[339] or Marimastat[340] to block these ECM changes.

In the present thesis, the initial trauma was provided by recombinant MMP-2, to test its ability to initiate similar proteolytic changes in the Wills model. These experiments were designed to test the theory that MMP-2 may bring about initiation of AAAs, as suggested by the data obtained in the previous two chapters. Brief, pre-treatment of segments of porcine aorta with pancreatic elastase or MMP-2 for 24hours, followed by culture for 14 days, was carried out to compare the action of these enzymes in the induction of medial elastolysis and to establish their ability to initiate aneurysm-like, degenerative changes. Tissue was analysed histologically, to determine the gross effects of the enzymes. Homogenates of the tissue were

also prepared to identify the influence of exogenous MMP-2 on intrinsic protease production by zymography.

The suggestion by Wills *et al* that the breakdown products of elastin were responsible for the induction of MMP production was also examined in this chapter. Previous work has suggested that EDPs may be chemotactic for inflammatory cells,[262] and EDPs are known to be present at elevated levels in the aorta[263] and urine[167] of AAA patients. Preliminary data has indicated that EDPs may also be involved in the activation of MMP-2, an interaction that may have ramifications for the development and progression of a vascular aneurysm. The presence of EDPs as a result of elastolysis may create a local positive feedback loop to activate more MMP-2 and thus degrade more elastin. Such a situation, combined with the chemotactic influence of EDPs for inflammatory cells, suggest that EDPs may be important in the pathogenesis of AAA.

To test the influence of EDPs on MMP activity, recombinantly produced MMP-2 was combined *in vitro* with EDPs prepared from elastase degraded elastin. Also, the effect of EDPs on the activity of secreted MMPs was tested by addition of EDPs to cultured cells and analysis of the culture medium.

Summary Of The Aims Of This Chapter

- To investigate the ability of MMP-2 to induce aneurysm-like changes in a model of AAA
- To examine the gross changes induced by MMP-2 pre-treatment of porcine aorta through histological examination
- To determine the effects of MMP-2 on porcine aorta by zymographic analysis of tissue homogenates
- To examine the role of elastin derived peptides upon MMP-2, both in recombinant form and that produced by cells *in vitro*

6.2 *The Influence Of MMP-2 In A Model Of Aneurysm Disease*

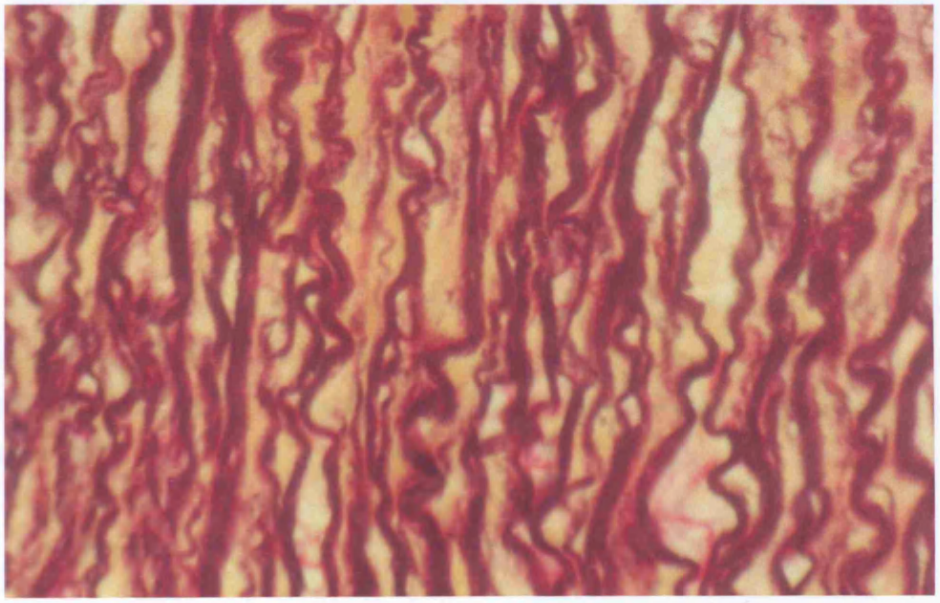
6.2.1 *Histological Analysis Of Porcine Aorta Specimens*

Porcine aorta was cultured for 14 days with and without the presence of pancreatic elastase or MMP-2, in experiments analogous to those carried out by Wills *et al.*[325] The enzyme was washed away thoroughly with culture medium, then replaced with fresh culture medium where it was incubated for 14 days with regular media replacement. Figure 6.1 illustrates the histological features of EVG stained porcine aorta, treated as described in the legend. The untreated control aorta exhibited distinct intimal, medial and adventitial layers, within which intact elastic lamellae were clearly defined (Figure 6.1a). This histological pattern was also observed in the elastase-treated aortas at day 1, but by day 4 the elastin fibres had begun to degenerate. By day 7 these changes had become more profound, and by the end of the culture period, the elastase had caused significant destruction of the aortic media, in a fashion very similar to that achieved by Wills (Figure 6.1b). Observations were identical in the MMP-2-treated aorta samples. Day one aortas exhibited intact elastic lamellae, but this progressively degenerated until day 14, when the elastic fibres were fragmented, with visible loss of medial integrity (Figure 6.1c). The degeneration of elastin was less profound in these experiments than that in Wills' study due to the reduced amount of enzyme used, particularly of recombinant MMP-2.

Visual scoring of the degradation of elastin in the aortas examined was performed, using a standard three point system to assess the ability of the pre-treatment enzymes to initiate ECM degeneration. Table 6.1 illustrates the results of this analysis, indicating that both elastase and MMP-2 pre-treatment yielded significantly elevated levels of elastin breakdown compared to non-pre-treated controls. These data further supported the hypothesis that MMP-2 was able to initiate degenerative changes which were analogous to those observed in AAAs.

Figure 6.1 **Elastin Van Gieson stained transverse sections of porcine aorta, following pre-treatment and 14 days in culture (x320). (a) Untreated, control porcine aorta, illustrating intact elastic lamellae. (b) Elastase (100U for 24hours) pre-treated porcine aorta, exhibiting fragmented elastin fibres. (c) MMP-2 (100ng/ml for 24hours) pre-treated porcine aorta, demonstrating the capacity for MMP-2 to induce degenerative changes in elastin fibres characteristic of aneurysm disease.**

(a)



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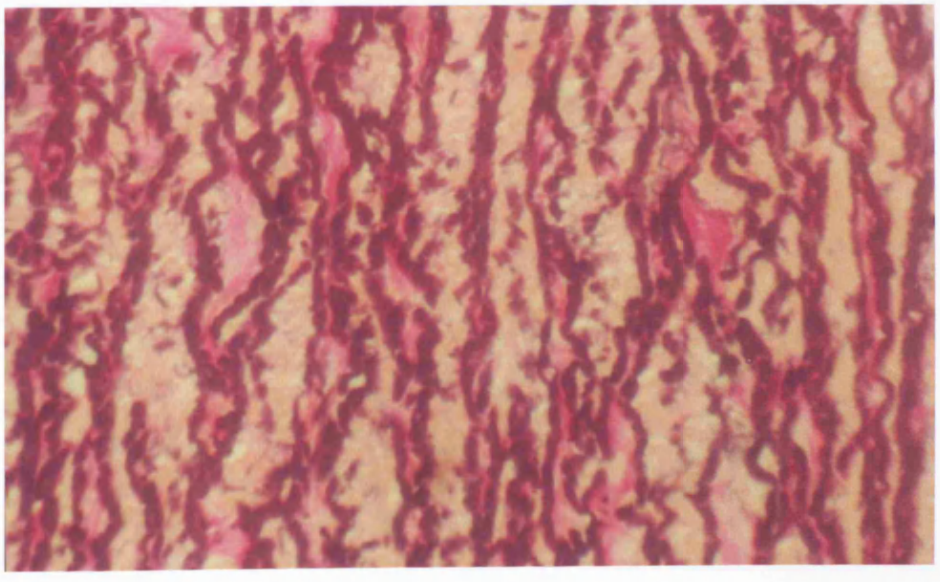

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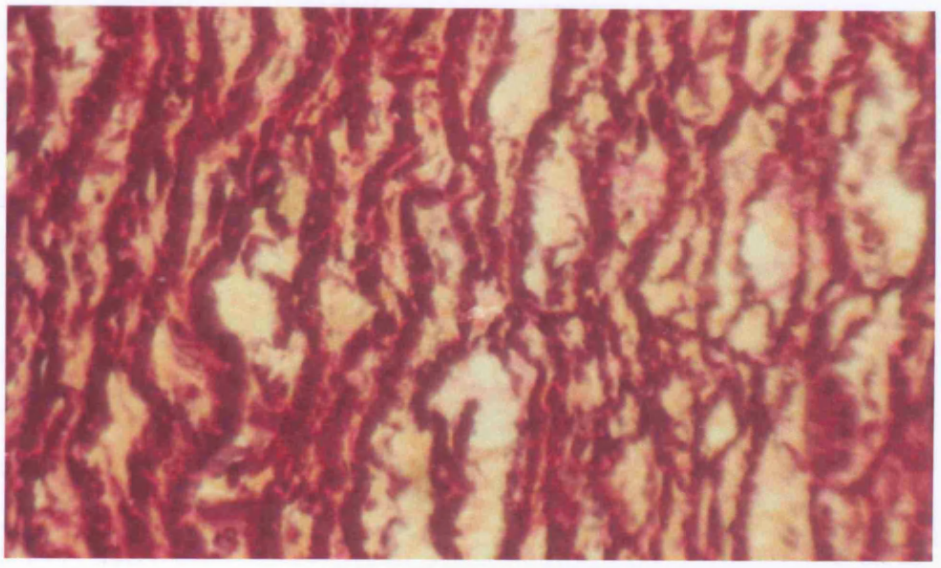


Table 6.1 **Summary of results of visual scoring of elastin degradation, based on a three point system where +/- was no fragmentation and +++ was strong fragmentation. These data illustrated the similar capacity of elastase and MMP-2 pre-treatment to initiate changes characteristic of AAAs.**

	Pre-treatment		
	Control	Elastase	MMP-2
Aorta 1	+/-	++	++
2	+	+++	+++
3	+/-	++	++
4	+/-	+++	++
5	+/-	++	+++
6	+/-	++	++
7	+	++	++
8	+/-	++	+++
9	+/-	++	++
10	+	++	+++
11	+	+++	++
12	+/-	++	++
13	+	++	++
14	+/-	++	+++

6.2.2 *Gelatin Zymography Analysis Of Porcine Aorta Homogenates*

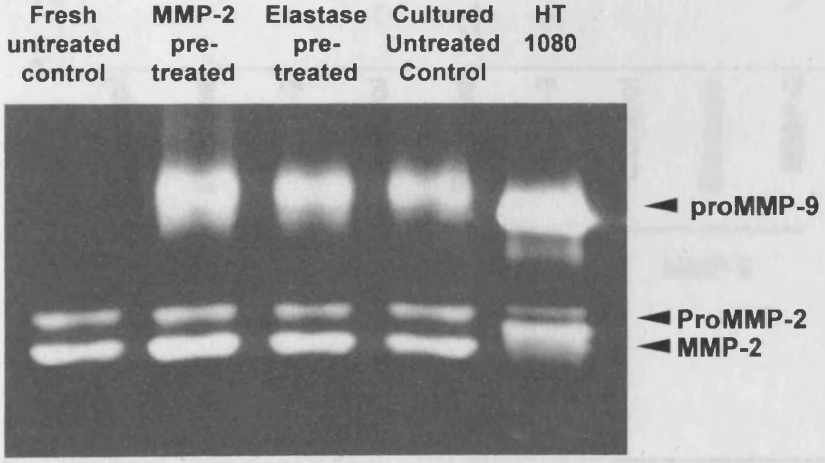
The results of the histological studies suggested that exogenous MMP-2 was capable of initiating elastolytic changes characteristic of the aneurysmal aorta. It was unclear, however, whether the exogenous MMP-2 was the sole cause of elastolysis or whether its presence had induced expression of other elastases, such as endogenous MMP-2 and 9. To confirm the pattern of such expression, homogenates of the aortic tissue were subjected to gelatin zymography. Figure 6.2 shows a typical zymogram, revealing production of proMMP-2, MMP-2 and proMMP-9 in the porcine aortas treated with both elastase and MMP-2 and untreated cultured tissue, as compared to the fresh, untreated control tissue, which contained only pro and active MMP-2. No other gelatinolytic activity was detected.

Scanning densitometry of these zymogram gels suggested that the amount of MMP-9 produced by the MMP-2-treated aortas was significantly higher than that produced by the untreated controls, as determined by Wilcoxon's analysis ($n=28$, $W=97.00$, $p=0.0009$). The amount of proMMP-9 in the elastase-treated aortas was also elevated above that in the untreated controls ($W=71.00$, $p=0.0245$), as suggested by the data of Wills *et al.* Similar analysis demonstrated that the amounts of proMMP-2 ($W=19.00$, $p=0.5830$) and MMP-2 ($W=51.00$, $p=0.1189$) were not significantly different between untreated controls and MMP-2-treated aortas.

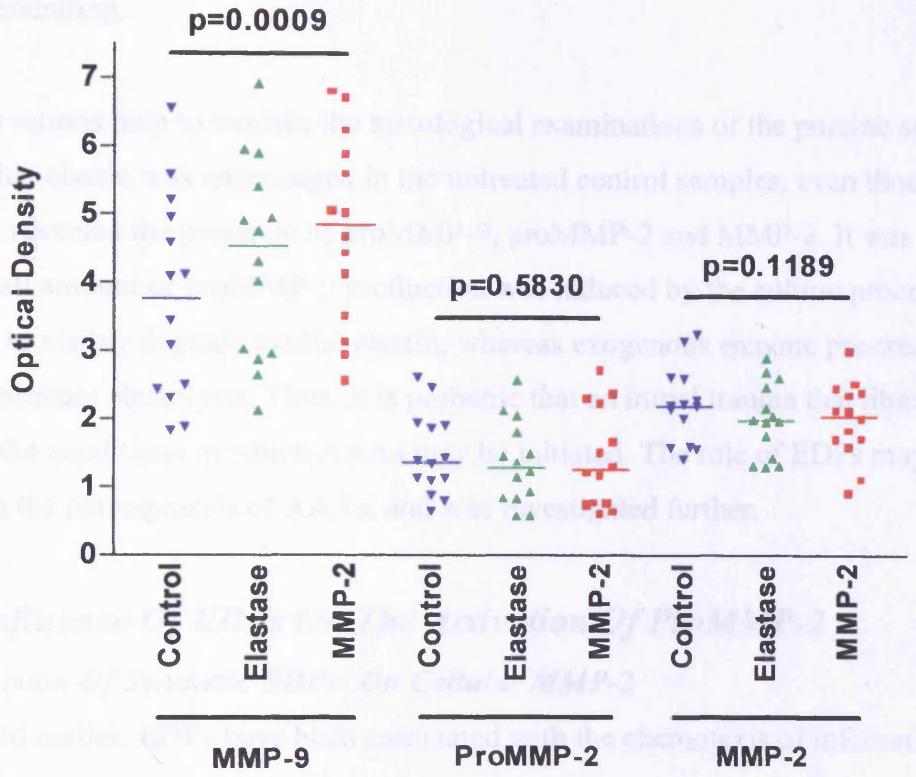
Western analysis of the porcine aortic homogenates was not possible, as the antibodies used previously did not readily detect the porcine MMPs or TIMPs, and no porcine-specific alternatives were available. Therefore, whilst it remained to be confirmed, the data from the zymography suggested that the cells of the porcine aortic wall were not induced to produce MMPs capable of degrading collagen. This may further strengthen the argument that collagen is degraded by MMPs secreted by cells other than those of the aortic wall, such as inflammatory cells, and therefore that the collagenolytic process, and the leukocytes, appears later in the disease.

Figure 6.2 **(a) Gelatin zymogram of homogenates of porcine aortas following pre-treatment and culture for 14 days. Homogenates were equalised for protein concentration prior to loading. The figure illustrates the increased production of proMMP-9 in aortas pre-treated with MMP-2 and elastase, compared to untreated tissue and fresh, uncultured tissue. Levels of MMP-2 were not significantly altered. (b) Scattergram of scanning densitometry data from zymographic analysis of porcine aorta homogenates, illustrating median values with p values. Statistical calculations compared the values from control and MMP-2 treated samples only. (c) Summary of scanning densitometry data, expressed as median optical density readings with 95% confidence intervals and p values.**

(a)



(b)



(c)

	Median optical density with 95% confidence intervals				
	Fresh Uncultured	Untreated Control	Elastase Treated	MMP-2 Treated	p Value
proMMP-9	-	3.760 (2.888-4.611)	4.526 (3.495-5.171)	4.846 (3.951-5.558)	0.0009
proMMP-2		1.358 (1.118-1.815)	1.286 (1.001-1.702)	1.250 (1.001-1.831)	0.5830
MMP-2		2.185 (1.864-2.516)	1.975 (1.676-2.296)	2.033 (1.645-2.294)	0.1189

The data collected from these experiments suggested that exogenous MMP-2, like pancreatic elastase, was capable of inducing profound degeneration of elastin fibres within the intact aorta. This was achieved by inducing MMP production in the cells of the porcine aortic tissue, via a mechanism which remains unresolved. However, it is likely that exogenous enzyme-mediated breakdown products of elastin damage, EDPs, were liberated into the aortic wall, causing upregulation of MMP production in the local SMCs. Subsequent removal of the

exogenous enzyme permitted observation of the subsequent role of endogenous MMPs in medial degeneration.

These observations help to explain the histological examinations of the porcine aortas, which suggested that elastin was undamaged in the untreated control samples, even though the zymograms revealed the presence of proMMP-9, proMMP-2 and MMP-2. It was apparent that whilst a small amount of proMMP-9 production was induced by the culture process, this was insufficient to visibly degrade medial elastin, whereas exogenous enzyme pre-treatment induced significant elastolysis. Thus, it is probable that an initial trauma that liberates EDPs may create the conditions in which AAAs may be initiated. The role of EDPs may therefore be crucial in the pathogenesis of AAAs, and was investigated further.

6.3 *The Influence Of EDPs On The Activation Of ProMMP-2*

6.3.1 *The Action Of Synthetic EDPs' On Cellular MMP-2*

As mentioned earlier, EDPs have been associated with the chemotaxis of inflammatory cells and the presence of AAAs, where it has been suggested they are a marker of disease. Preliminary studies by a colleague had further suggested that EDPs may activate proMMP-2 directly, which may have considerable influence on the developing aneurysm *in vivo*. To examine the latter hypothesis, experiments were carried out using synthetic EDPs, generated *in vitro* by the digestion of elastin with porcine pancreatic elastase.

EDPs were added to fixed numbers of saphenous vein SMCs in culture, to determine their effect on the production and activation of MMPs by the cells. Media was collected from the cells following a 48hour period of exposure. Figure 6.3 shows a gelatin zymogram of samples taken from such treated cells. Media from untreated cells contained only proMMP-2, as previously demonstrated in control aorta and aneurysm-derived SMCs. However, as the concentration of EDPs added to them was increased, the proMMP-2 produced by the cells was activated in a dose-dependent manner. Scanning densitometry of the zymogram gels showed that this increase in activation was statistically significant according to Kruskal Wallis analysis (Figure 6.4). Furthermore, the usual appearance of proMMP-2 at 72kDa and active MMP-2 at around 60kDa, were accompanied by smaller isoforms of active MMP-2 at around 55, 50 and 40kDa as the concentration of EDPs increased. These isoforms may represent unusual variants of the enzyme, which had been processed to an advanced state by autolysis

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as described in Chapter One. Controls, including elastase and elastin alone, exhibited no activation of proMMP-2, suggesting that this phenomenon was mediated by the EDPs. The mode of this action was not clear, but may represent a direct effect upon the secreted enzymes, or reflect interaction or uptake of the EDPs with the cells.

Figure 6.3 (a) Gelatin zymogram of media samples from saphenous vein SMCs stimulated with varying concentrations of EDPs. ProMMP-2 activation appears to occur in a dose-dependent manner according to EDP concentration. Numerous isoforms of active MMP-2 became apparent at higher concentrations of EDPs, suggesting advanced processing of the MMP-2 molecule.

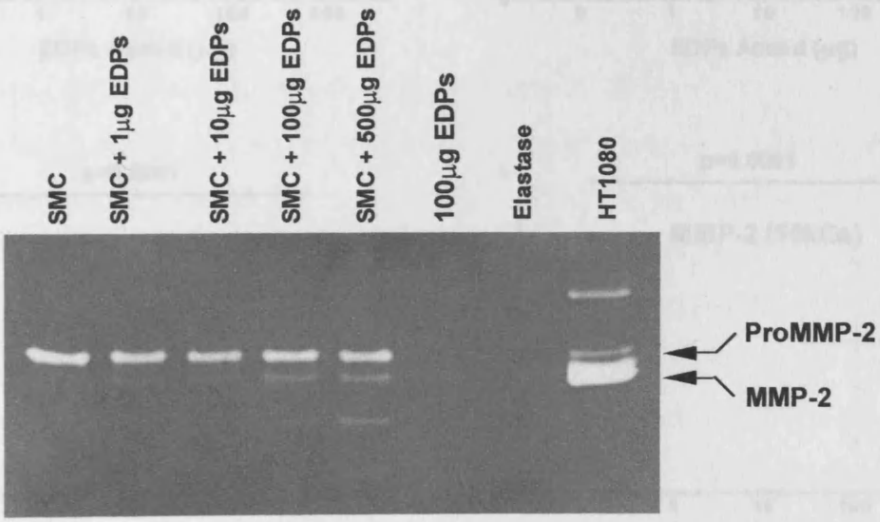
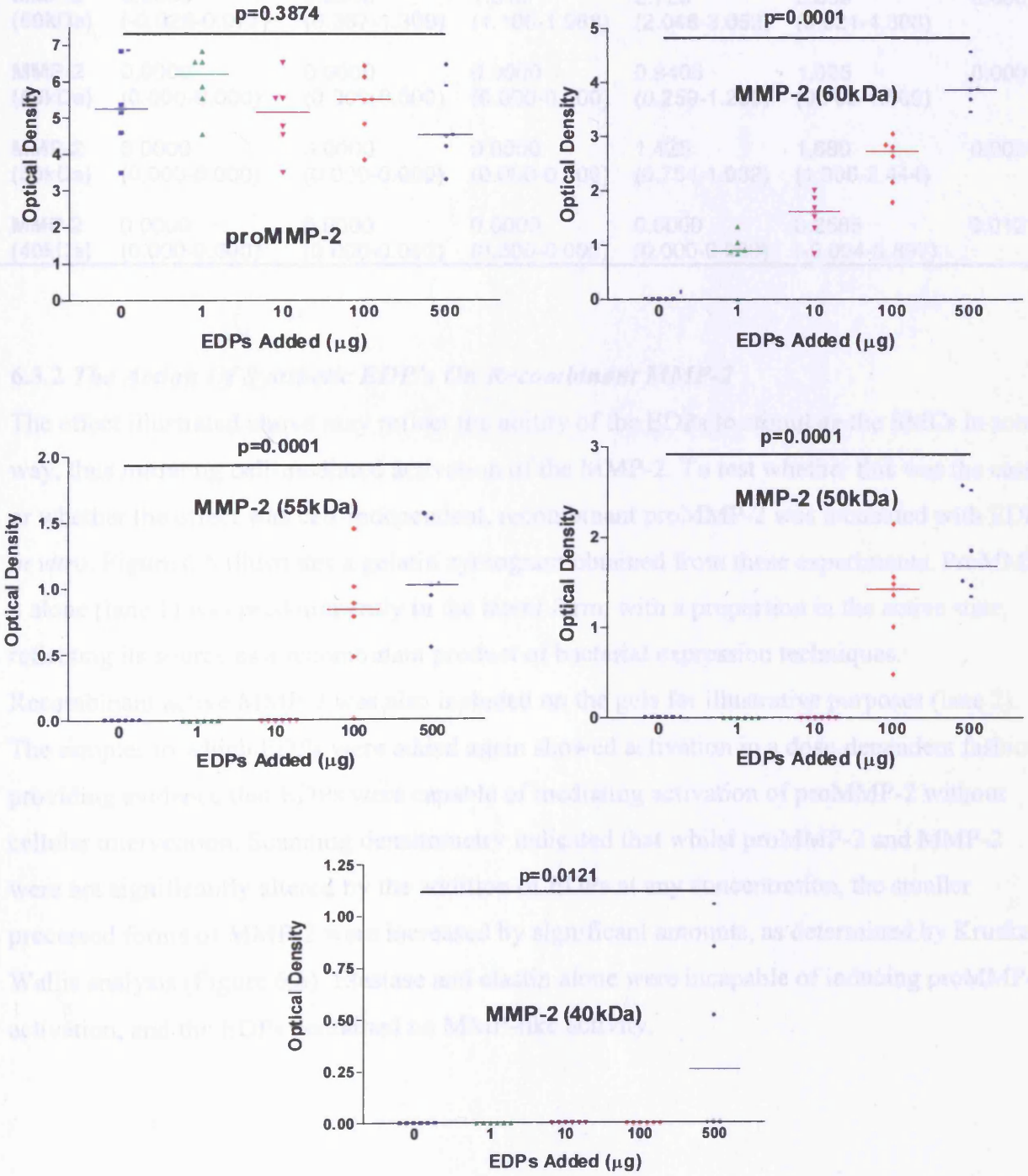


Figure 6.4 (a) Scattergrams of scanning densitometry data from EDP treatment of SMCs, showing median optical densities and p values. (b) Summary of scanning densitometry data, expressed as median values with 95% confidence intervals and p values (Kruskal Wallis).

(a)



(b)

Band	Median optical density with 95% confidence intervals At given EDP concentration					p Value
	No EDPs	1μg EDPs	10μg EDPs	100μg EDPs	500μg EDPs	
Pro MMP-2	5.261 (4.003-6.761)	6.220 (5.102-6.889)	5.175 (3.998-6.181)	5.531 (4.417-6.379)	4.539 (3.616-6.037)	0.3484
MMP-2 (60kDa)	0.0000 (-0.028-0.077)	0.8960 (0.367-1.309)	1.613 (1.106-1.988)	2.725 (2.046-3.052)	3.859 (3.521-4.308)	0.0001
MMP-2 (55kDa)	0.0000 (0.000-0.000)	0.0000 (0.000-0.000)	0.0000 (0.000-0.000)	0.8405 (0.259-1.289)	1.025 (0.702-1.509)	0.0001
MMP-2 (50kDa)	0.0000 (0.000-0.000)	0.0000 (0.000-0.000)	0.0000 (0.000-0.000)	1.425 (0.754-1.932)	1.680 (1.306-2.444)	0.0001
MMP-2 (40kDa)	0.0000 (0.000-0.000)	0.0000 (0.000-0.000)	0.0000 (0.000-0.000)	0.0000 (0.000-0.000)	0.2565 (-0.094-0.897)	0.0121

6.3.2 *The Action Of Synthetic EDP's On Recombinant MMP-2*

The effect illustrated above may reflect the ability of the EDPs to stimulate the SMCs in some way, thus initiating cell-mediated activation of the MMP-2. To test whether this was the case, or whether the effect was cell-independent, recombinant proMMP-2 was incubated with EDPs *in vitro*. Figure 6.5 illustrates a gelatin zymogram obtained from these experiments. ProMMP-2 alone (lane 1) was predominantly in the latent form, with a proportion in the active state, reflecting its source as a recombinant product of bacterial expression techniques. Recombinant active MMP-2 was also included on the gels for illustrative purposes (lane 2). The samples to which EDPs were added again showed activation in a dose-dependent fashion, providing evidence that EDPs were capable of mediating activation of proMMP-2 without cellular intervention. Scanning densitometry indicated that whilst proMMP-2 and MMP-2 were not significantly altered by the addition of EDPs at any concentration, the smaller processed forms of MMP-2 were increased by significant amounts, as determined by Kruskall Wallis analysis (Figure 6.6). Elastase and elastin alone were incapable of inducing proMMP-2 activation, and the EDPs contained no MMP-like activity.

Figure 6.5 Gelatin zymogram of reaction products from the incubation of recombinant proMMP-2 with varying concentrations of EDPs. Activation of proMMP-2 appeared to occur in a dose-dependent fashion with increasing concentration of EDPs. Multiple isoforms of active MMP-2 became apparent with increasing EDP concentration.

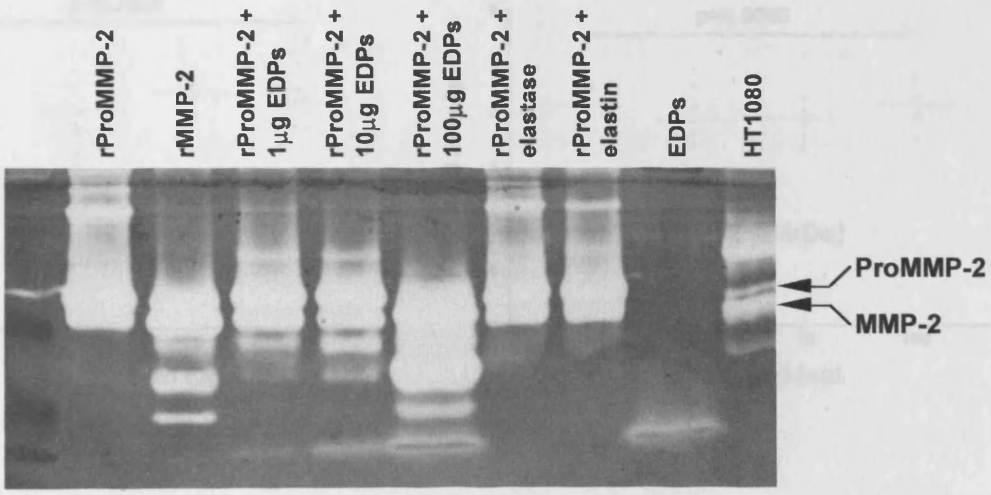
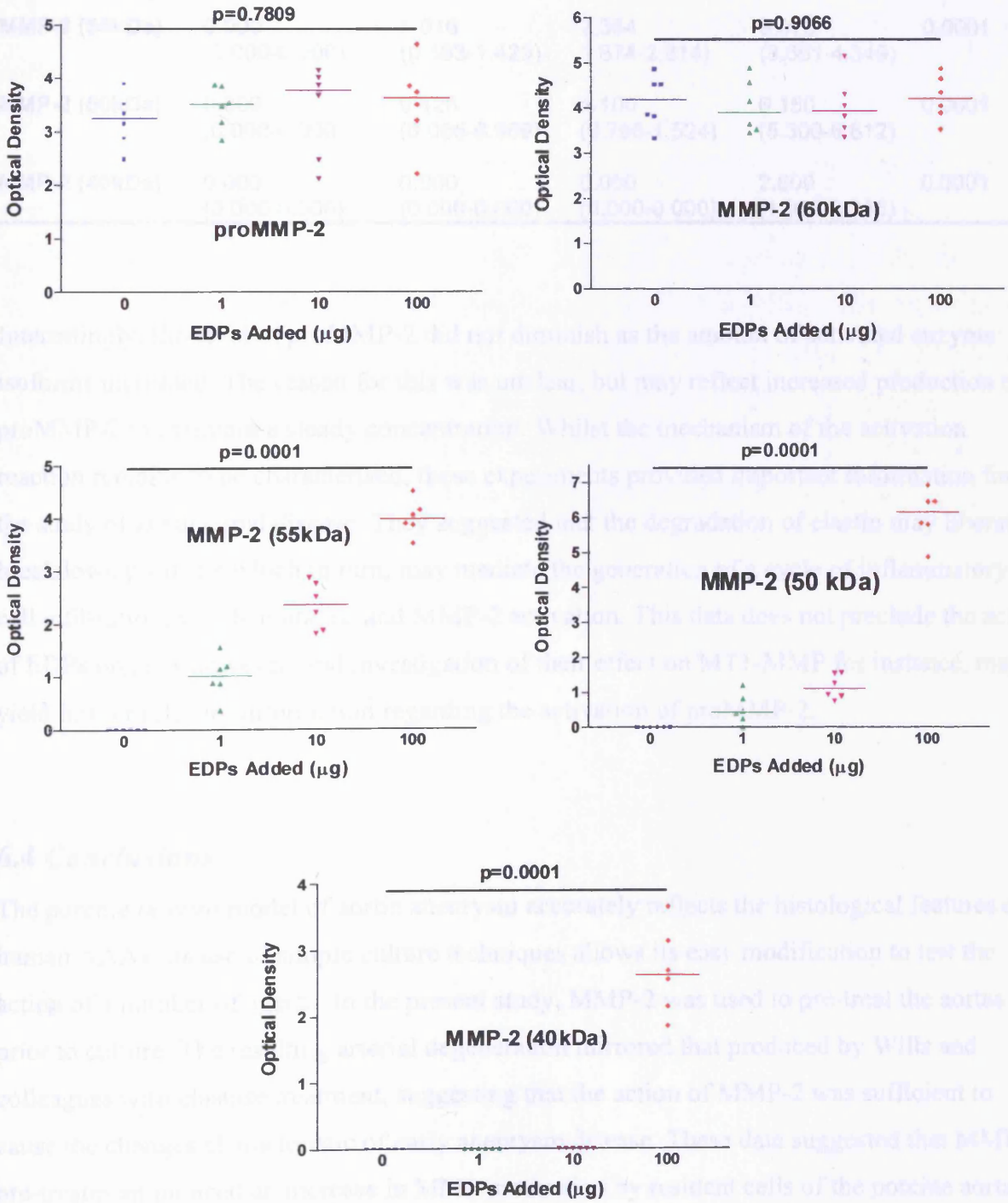


Figure 6.6 (a) Scattergrams of scanning densitometry data, indicating median optical densities of individual zymogram bands at particular EDP concentrations with p values. (b) Summary of scanning densitometry data expressed as median values with 95% confidence intervals and p values (Kruskal Wallis).

(a)



(b)

Band	Median optical density with 95% confidence intervals at given EDP concentration				p Value
	No EDPs	1μg EDPs	10μg EDPs	100μg EDPs	
proMMP-2	3.253 (2.696-3.731)	3.335 (3.046-3.887)	3.081 (2.483-4.299)	3.367 (2.691-4.379)	0.7809
MMP-2 (60kDa)	4.181 (3.536-4.744)	3.901 (3.415-4.670)	3.938 (3.370-4.714)	4.201 (3.704-4.750)	0.9066
MMP-2 (55kDa)	0.000 (0.000-0.000)	1.016 (0.583-1.429)	2.364 1.874-2.814)	3.976 (3.651-4.349)	0.0001
MMP-2 (50kDa)	0.000 (0.000-0.000)	0.426 (0.065-0.969)	1.100 (0.795-1.524)	6.150 (5.300-6.812)	0.0001
MMP-2 (40kDa)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	0.000 (0.000-0.000)	2.609 (1.976-3.345)	0.0001

Interestingly, the level of proMMP-2 did not diminish as the amount of activated enzyme isoforms increased. The reason for this was unclear, but may reflect increased production of proMMP-2 to maintain a steady concentration. Whilst the mechanism of the activation reaction remains to be characterised, these experiments provided important information for the study of aneurysmal disease. They suggested that the degradation of elastin may liberate breakdown products which in turn, may mediate the generation of a cycle of inflammatory cell infiltration, via chemotaxis, and MMP-2 activation. This data does not preclude the action of EDPs on cells however, and investigation of their effect on MT1-MMP for instance, may yield further relevant information regarding the activation of proMMP-2.

6.4 *Conclusions*

The porcine *in vitro* model of aortic aneurysm accurately reflects the histological features of human AAAs. Its use of simple culture techniques allows its easy modification to test the action of a number of agents. In the present study, MMP-2 was used to pre-treat the aortas prior to culture. The resulting arterial degeneration mirrored that produced by Wills and colleagues with elastase treatment, suggesting that the action of MMP-2 was sufficient to cause the changes characteristic of early aneurysm disease. These data suggested that MMP-2 pre-treatment induced an increase in MMP production by resident cells of the porcine aorta, which brought about elastin destruction. This evidence, combined with the observation that

MMP-2 is the most prominent elastolytic enzyme in small aneurysms, lends weight to the hypothesis that MMP-2 may be capable of the initiation of AAAs.

Furthermore, the consequences of elastolysis may complicate the local proteolytic and inflammatory environment within the nascent aneurysm. The results presented in the latter half of the present chapter suggested that the liberation of EDPs in the degenerating aortic wall may directly activate proMMP-2 produced by the resident SMCs. This phenomenon may contribute to ECM catabolism and exacerbate inflammation

When combined with the data presented in the previous chapter, the findings described here form the beginnings of a new model of aneurysm initiation and progression. Namely, the medial SMCs may be the source of elevated levels of proMMP-2, which in combination with SMC-derived MT1-MMP and TIMP-2, bring about progressive destruction of aortic elastin fibres. The breakdown of these fibres may liberate EDPs, with concomitant activation of local MMP-2, and further elastin degradation. Also, EDPs may attract inflammatory cells, which liberate further MMP activity, including the collagenolytic enzymes which correlate with aortic dilatation. This model is hypothetical and will be developed further in the discussion of this thesis.

CHAPTER SEVEN

Results:

Dermal Fibroblasts

7.1 *Introduction*

The work described in Chapter Five provided strong evidence of an increase in the production of MMP-2 by vascular SMCs in AAA patients compared to control cells, which appeared to reflect a primary difference between the cells. Chapter Six supported the theory that elevated amounts of MMP-2 may contribute to elastin degradation and hence aneurysmal degeneration of the aorta. Together, the findings of these two chapters provided the beginnings of a model of aneurysmal disease and implicated medial SMCs in the genesis of AAAs.

Contemporary evidence has stated that the SMCs of the aorta are heterogeneous in their embryological origins.[341] During morphogenesis of the vascular system, cells derived from the mesoderm migrate to form 'blood islands', clusters of mesenchymal cells which then differentiate according to their location. Cells on the periphery of the islands become endothelial cells, and hence the lining of the vasculature, whereas the central cells become the nucleated blood cells such as leukocytes.[342] Further mesenchymal cells then differentiate to 'flesh out' the outer layers of the primitive vessels, such as SMCs. In addition to this, studies have shown that endothelial cells may transdifferentiate into SMCs.[343] Whilst this represents an alternative source for arterial SMCs, the endothelial cells were originally derived from mesoderm, and thus the SMCs may be said to have the same lineage as those which directly acquired smooth muscle characteristics.

These events beget the majority of the systemic vasculature, including the descending aorta.[344] However, it has also been demonstrated, particularly by lineage tracing of cells in chick embryos, that the SMCs of the aortic arch, pulmonary artery arch and carotid arteries are derived from neural crest.[345] This is a specialised subset of ectodermal cells, called neuroectoderm, which also yield the central nervous system, the cranial sensory ganglia, the adrenals and pigment cells.

These reports suggested that the abdominal aortic SMCs studied in previous chapters of this thesis were mesenchymal in origin. Thus, a hypothesis was formed to suggest that differences observed in these aortic SMCs may be present in other tissues with mesodermal origins. Such tissues include muscle, cartilage, bone, dermis and the urino-genital system.[342]

To test this hypothesis, skin biopsies were obtained from patients with AAAs and from control patients during surgery. The demographics of these patients are summarised in Table 7.1. Biopsies were taken from the abdominal skin exposed by the surgical incision. These biopsies were used to derive dermal fibroblast cell lines, known to be of mesodermal origin,[346] and these cells were used in comparative studies in which their MMP production was assessed. The data obtained from SMCs suggested that MMP-2 production may also be elevated in the dermal fibroblasts of patients with AAAs as compared to those of controls. Conditioned media was collected from all cell lines, and used in gelatin zymography, western blotting and ELISA analysis of MMP and TIMP production. RNA was also extracted from the cells, and used in northern analysis of gene expression. Having established that the differences in SMCs were confined to elevated MMP-2 expression, only this enzyme and its associated activator and inhibitor, MT1-MMP and TIMP-2 respectively, were examined in this chapter. Conformation of this hypothesis by detection of increased MMP-2 expression in the cells of the skin, may for example, permit the development of a biopsy-based test for aneurysm predisposition.

Summary Of The Aims Of This Chapter

- **To investigate the hypothesis that other mesenchyme-derived cells in AAA patients display increased MMP-2 production**
- **To derive dermal fibroblast cell lines from skin biopsies taken from control and AAA patient groups**
- **To compare the production of MMP-2 in these cell lines by gelatin zymography**
- **To compare the production of MMPs and TIMPs by these cell lines by ELISA**
- **To compare the expression of MMP-2, MT1-MMP and TIMP-2 in these cell lines by northern blotting**

7.2 *MMP And TIMP Production By Dermal Fibroblasts*

7.2.1 *Gelatin Zymography Analysis Of Dermal Fibroblast Culture Media Samples*

32 cell lines were established from skin biopsies taken from the abdomen of the patients listed in Chapter Five, plus another 18 patients from whom aortic tissue was not obtained. The demographics of the patients from whom tissue samples were obtained are described in Table 7.1. Control patients all had evidence of peripheral atherosclerosis, and all patients were either non or ex-smokers. Analysis of the patient groups suggested that the median ages between controls and patients with AAAs were not significantly different (control median age 68.5 (64.72-70.15), AAA median age 69.0 (67.04-73.46); n=14, U=11.00, p=0.2742), as established by Mann-Whitney analysis.

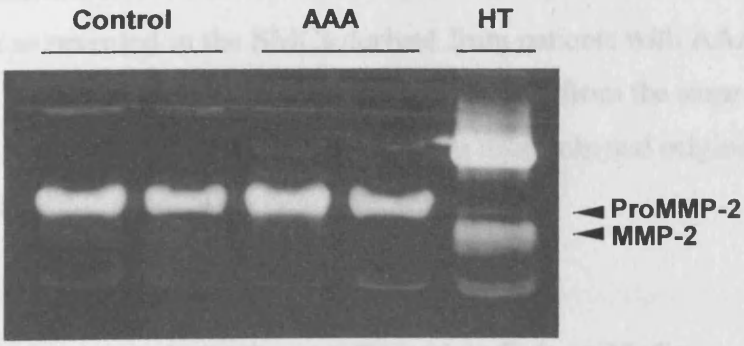
Table 7.1 Demographics of patients from whom skin biopsies were obtained for dermal fibroblast studies.

Patient	Age	Sex	Aortic Diameter (cm)
Control 1	58	M	<2.5
Control 2	67	M	<2.5
Control 3	75	F	<2.5
Control 4	75	M	<2.5
Control 5	70	M	<2.5
Control 6	64	F	<2.5
Control 7	68	M	<2.5
Control 8	72	M	<2.5
Control 9	69	M	<2.5
Control 10	69	F	<2.5
Control 11	58	F	<2.5
Control 12	70	M	<2.5
Control 13	64	F	<2.5
Control 14	66	M	<2.5
Control 15	71	M	<2.5
Control 16	63	M	<2.5
AAA 1	81	M	7.7
AAA 2	72	M	6.0
AAA 3	67	M	6.0
AAA 4	78	M	7.0
AAA 5	77	M	9.0
AAA 6	77	M	5.5
AAA 7	70	M	7.0
AAA 8	68	M	6.5
AAA 9	71	F	7.5
AAA 10	76	M	7.0
AAA 11	64	M	8.5
AAA 12	67	F	7.5
AAA 13	61	M	6.5
AAA 14	65	M	8.0
AAA 15	63	M	9.0
AAA 16	67	M	6.5

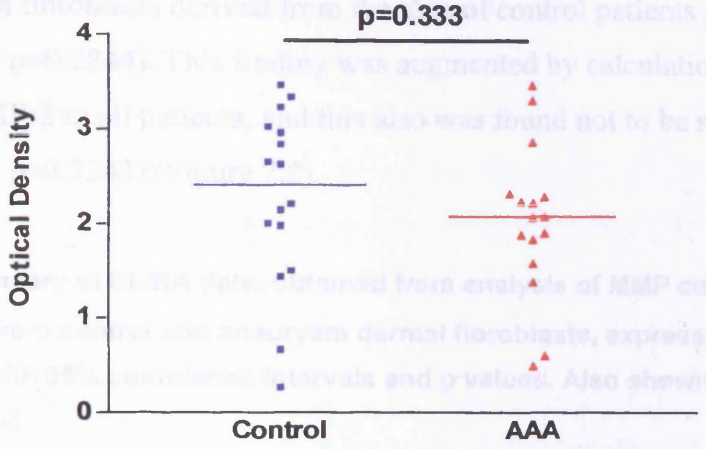
Serum free conditioned media was collected from 10^5 cells of each cell line and subjected to gelatin zymography, ELISA and western analysis to determine their MMP/TIMP content. Gelatin zymography of conditioned media samples revealed that like SMCs, the dermal fibroblasts produced only proMMP-2 into the medium. No other gelatinolytic activity was detected on the gels. However, unlike SMCs the proMMP-2 production of dermal fibroblasts derived from control and AAA skin biopsies was not significantly different, as determined by Wilcoxon's analysis of the scanning densitometry data (n=32, W=38.00, p=0.333) (Figure 7.1).

Figure 7.1 (a) Gelatin zymogram of duplicate conditioned media samples from control and AAA dermal fibroblasts, illustrating their similar levels of proMMP-2 production. Media was conditioned for 48hrs by 10^5 cells. Media obtained from HT-1080 fibrosarcoma cells was included as a positive control. (b) Scattergram of median densitometry readings, with p values indicated. (c) Summary of scanning densitometry data, indicating median values with 95% confidence intervals and p value.

(a)



(b)



(c)

Median optical density with 95% confidence intervals		
Control	**AAA**	**p Value**
2.42 (1.77-2.77)	2.078 (1.61-2.46)	0.333

These data suggested that the fibroblasts did not carry the same predisposition to elevated proMMP-2 production as revealed in the SMCs derived from patients with AAAs. This evidence may indicate that although the fibroblasts were derived from the same embryological source, their pattern of differentiation from mesenchymal origins had resulted in different control of MMP-2 production than SMCs.

7.2.2 *Western And ELISA Analysis Of Dermal Fibroblast Culture Media*

Western blotting was employed to confirm the identity of the MMP activity detected by zymography. All cultures produced MMP-2, TIMP-1 and TIMP-2-reactive material, consistent with previous findings with SMCs, but no other MMP activity was revealed by this method. Data not shown. Quantitative analysis of these media samples by MMP/TIMP ELISA tests confirmed the findings suggested by gelatin zymography, as summarised in Figure 7.2. As with the SMCs, the dermal fibroblasts were found to produce large amounts of MMP-2 and TIMP-1, but little MMP-1, 3 or 9 and again, surprisingly, little TIMP-2. As before, measurement of MT1-MMP levels was not possible using this methodology.

However, unlike the aortic SMCs, the level of MMP-2 was not found to be significantly different between fibroblasts derived from the skin of control patients and patients with AAAs (n=32, U=12.00, p=0.2844). This finding was augmented by calculation of the ratio between MMP-2 and TIMP-2 in all patients, and this also was found not to be significantly different (n=32, U=17.00, p=0.7242) (Figure 7.2).

Figure 7.2 Summary of ELISA data, obtained from analysis of MMP content of conditioned culture medium from control and aneurysm dermal fibroblasts, expressed as median concentrations with 95% confidence intervals and p values. Also shown is the ratio between MMP-2 and TIMP-2.

	Median concentration (ng/ml) with 95% confidence intervals		
	Control	**AAA**	**p value**
MMP-1	63.06 (0-138.3)	19.38 (7.68-70.96)	0.364
MMP-2	779.6 (601.0-1097.0))	662.5 (473.8-1056.0)	0.284
MMP-3	35.02 (17.55-62.74)	21.28 (12.85-31.23)	0.045
MMP-9	113.1 (66.37-152.2)	107.0 (64.38-132.4)	0.724
TIMP-1	599.1 (395.2-795.6)	426.2 (337.4-540.2)	0.147
TIMP-2	19.44 (10.88-24.72)	14.46 (12.01-21.07)	0.797
MMP-2: TIMP-2 Ratio	60.52 (27.12-77.50)	45.73 (30.12-67.02)	0.7242

7.3 *MMP And TIMP Gene Expression By Dermal Fibroblasts*

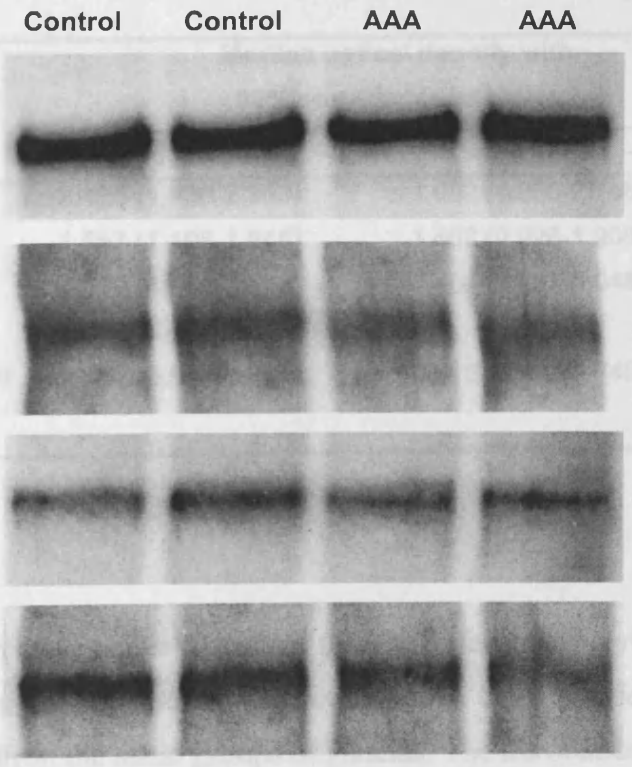
The previous studies in this chapter had indicated that proMMP-2 production was not significantly different between dermal fibroblasts derived from patients with AAAs and controls. Thus, it was important to confirm that these findings were echoed at the level of gene expression. Transcription of the MMP-2, TIMP-2 and MT1-MMP genes was confirmed by RT-PCR detection of mRNAs, as before (not shown). Comparison of the relative levels of gene expression was assessed by northern blotting.

As with the findings for SMCs, the data obtained from scanning densitometry of the northern blots reflected that collected from the zymography and ELISA tests. Figure 7.3 demonstrates a

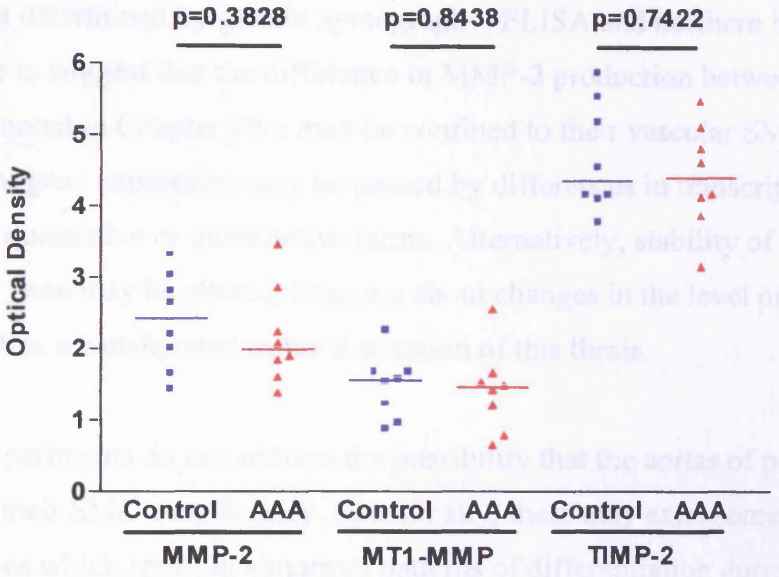
typical northern blot autoradiogram, indicating the similar level of expression of the MMP-2 gene between the control and AAA fibroblast cell lines. Wilcoxon's analysis of the densitometry data indicated that the expression of MMP-2 was not significantly different between fibroblasts derived from patients with AAAs and controls (n=32, W=14.00, p=0.3828). Similar results were obtained when probing for TIMP-2 and MT1-MMP. Scanning densitometry of the blots suggested that the level of expression of MT1-MMP (p=0.8438) and TIMP-2 (p=0.7422) were not significantly different between the control and aneurysm fibroblasts.

Figure 7.3 **(a) Northern blot of control and aneurysmal dermal fibroblast RNA (5μg) probed as indicated, illustrating a similar level of expression. (b) Scattergram of northern blot scanning densitometry data, with medians and p values indicated. (c) Summary of scanning densitometry data expressed as median values with 95% confidence intervals.**

(a)



(b)



(c)

	Median optical density with 95% confidence intervals		
	Control	**AAA**	**p Value**
MMP-2	2.426 (1.830-2.953)	1.992 (1.600-2.746)	0.3828
MT1-MMP	1.557 (1.108-1.848)	1.457 (0.926-1.908)	0.8438
TIMP-2	4.359 (4.034-5.313)	4.404 (3.807-5.048)	0.7422
MMP-2:GAPDH Ratio	0.758 (0.513-0.864)	0.618 (0.481-0.743)	0.3125

7.4 *Conclusions*

The data obtained in this chapter suggested that the differences previously observed in MMP-2 expression between control and AAA vascular SMCs was not reflected in dermal fibroblasts derived from the same groups of patients. The fibroblasts were known to be embryologically derived from the same mesodermal source as the aortic SMCs previously studied in this thesis. Therefore, the results obtained in this chapter suggested that, contrary to the hypothesis, a generalised difference in MMP-2 production was not common to all mesenchyme-derived tissue.

Dermal fibroblasts cultured from control patients and those with aneurysms did not exhibit any significant difference in their production or expression of MMP-2, TIMP-2 or MT1-MMP, as determined by gelatin zymography, ELISA and northern blotting. This provided evidence to suggest that the difference in MMP-2 production between control and aneurysm patients noted in Chapter Five may be confined to their vascular SMCs. Such tissue-specific control of gene expression may be caused by differences in transcription factor binding events in either qualitative or quantitative terms. Alternatively, stability of the mRNA transcribed from the gene may be altered, bringing about changes in the level of protein translated. Such possibilities are elaborated in the discussion of this thesis.

These experiments do not address the possibility that the aortas of patients with AAAs may differ in their SMC constituency. Specifically, there may exist some developmental differences which result in abnormal patterns of differentiation during morphogenesis of the vasculature. Previous experiments have shown that SMCs derived from neural crest and those from mesenchyme have differing responses to growth factors or to ECM components and integrins.[347,348] Such fundamental differences between the SMC types may result in altered physiological stability *in vivo*, for example under conditions of stress such as those in the wall of the atherosclerotic aorta. Future studies may involve the determination of the clonality of the SMCs and fibroblasts cultured, to determine their embryological source and to define the validity of these experiments.

Thus, the data in the present chapter suggest that the elevated levels of MMP-2 produced by aortic SMCs from AAA patients compared to controls were not a reflection of a generalised trait in all mesenchyme-derived tissues. Furthermore, they may suggest that this increase in MMP-2 expression is confined to vascular SMCs, or even specifically to the SMCs of the aorta. The findings of Ward[129] of increased dilatation throughout the vasculature of patients with AAAs may suggest increased proteolysis within the vessel walls. Thus, it may be possible to detect such changes in vascular tissue remote from the site of an aneurysm, which may merely be a manifestation of a generalised vascular process. To test this hypothesis, analysis of the MMP production within other vascular tissue was required to determine how these differences were distributed in the AAA patients. The following chapter addressed this question.

CHAPTER EIGHT

Results:

Inferior Mesenteric Vein

8.1 *Introduction*

The findings of Chapter Five provided evidence to suggest a predisposition to increased MMP-2 expression by aortic SMCs derived from patients with AAAs compared to controls, but results from dermal fibroblast culture experiments in the previous chapter suggested that this change was not found in all tissues derived from mesenchyme, and indeed, may be confined to their vascular tissue. The implication of these data, particularly when combined with the evidence for generalised vascular dilatation in AAA patients provided by Ward,[129] was that these patients may harbour a genetic alteration which may cause generalised vascular dilatation through increased ECM degradation. This phenomenon may be the result of a number of different contributory events, all of which have a common result which is the tendency towards gradual dilatation of the blood vessels. For example, this may be a product either of increased proteolysis of vessel architecture, or may reflect an underlying weakness in the make-up of that structure. Ward's findings suggested that as patients with AAAs possess a tendency for dilatation in their entire vasculature, there may be evidence of increased proteolysis within any vessel studied. Thus, a hypothesis was formed that the elevated MMP-2 expression observed in SMCs derived from aneurysmal aortas, may also be found in other parts of the vasculature of those patients.

The experiments described in this chapter aimed to investigate this hypothesis further, by examining the MMP production of an alternative source of vascular tissue, inferior mesenteric vein (IMV). This vessel is commonly removed during abdominal surgery, and was ideal for the purposes of this thesis. It is venous, rather than arterial, and represents a site of the vasculature remote from the abdominal aorta where aneurysmal degeneration occurs. It was hypothesised that the differences in relative levels of MMP-2 expression exhibited by aortic SMCs may be detected in the IMV samples, thus providing further evidence of a vascular tissue-specific alteration in the regulation of MMP-2 expression. This hypothesis was tested by analysing the IMV tissue histologically and by directly comparing the MMP content of the veins derived from patients with AAAs and those from control patients.

Summary Of The Aims Of This Chapter

- To test the hypothesis that elevated MMP-2 expression in AAA patients is present throughout the vascular system
- To prepare paraffin-wax blocks from formaldehyde-fixed blocks of IMV tissue, and perform histological examination of the samples to determine any gross differences between IMV from patients with AAAs versus controls
- To prepare whole tissue homogenates of the IMV samples
- To analyse the MMP content of the IMV homogenates by gelatin zymography and ELISA, to determine the nature and level of any differences revealed

8.2 *Patient Details*

IMV samples were obtained from patients undergoing elective repair of AAAs as previously described. Control IMV samples were obtained from a diverse group of patients, most of whom were undergoing corrective surgery for either colitis or diverticular disease of the colon. It was felt that neither of these conditions presented any difficulties in terms of their influence on MMP production within the tissue specimens. However, it is important to stress that none of the patients were suffering from any malignancies, which may have influenced the data obtained. The demographics of the patients involved in this study are summarised in Table 8.1.

Table 8.1 Demographics of patients from whom tissue was obtained for IMV studies.

	Sex	Aortic Diameter (cm)	Pathology
Control 1	M	<2.5	Colitis
Control 2	F	<2.5	Colitis
Control 3	M	<2.5	Diverticular
Control 4	M	<2.5	Colitis
Control 5	M	<2.5	Diverticular
Control 6	F	<2.5	Diverticular
Control 7	F	<2.5	Colitis
Control 8	M	<2.5	Colitis
AAA 1	F	7.7	AAA
AAA 2	M	6.0	AAA
AAA 3	M	6.0	AAA
AAA 4	F	7.0	AAA
AAA 5	M	9.0	AAA
AAA 6	M	5.5	AAA
AAA 7	M	7.0	AAA
AAA 8	M	6.5	AAA

IMV tissue was chosen for these studies as it was readily accessible during abdominal surgery such as AAA repair, provided a venous, rather than arterial perspective on the events studied, and was relatively remote from the aorta. It also contained the main elements studied in these experiments, namely SMCs and elastin, and as such represented a useful model for their examination. Unfortunately, only a small amount of IMV was available from surgery, which prevented the establishment of cell lines. Instead, whole tissue histological studies were made, and tissue homogenates were then analysed using zymography and western blotting techniques as before.

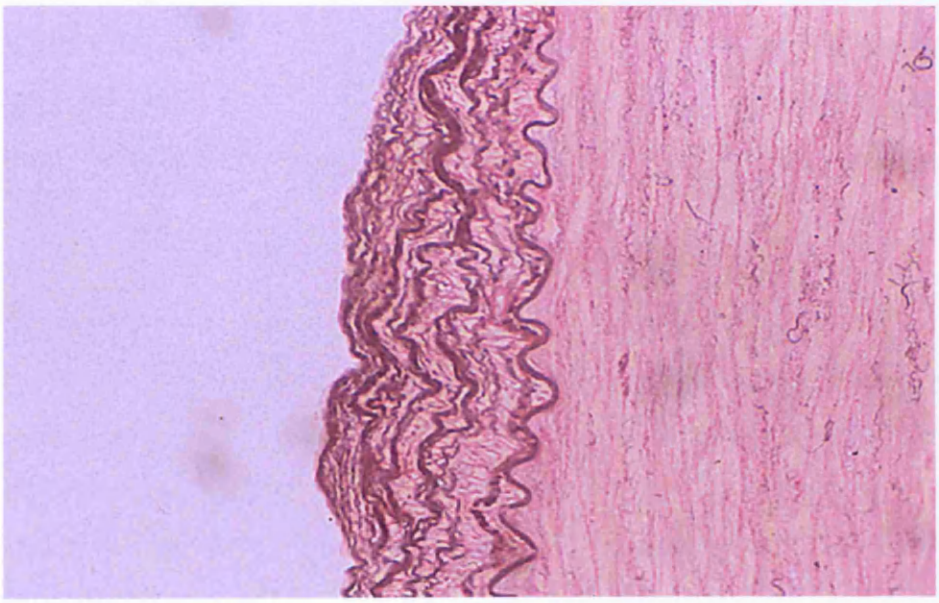
8.3 *Histological Studies*

Figure 8.1 illustrates EVG-stained sections of intact IMV sections. The tissue derived from control and aneurysm patients was markedly different. The most profound change was in the amount and integrity of elastin fibres. Control vein contained numerous circumferentially arranged elastin fibres in the medial and adventitial layers. In contrast, vein from aneurysm patients exhibited visibly reduced elastin content, the fibres of which were fragmented and incomplete throughout the section, along with a generalised disorganisation of the vessel architecture. Furthermore, collagen deposition was uniform in the control vein, reflecting the elastic lamellae characteristic of elastic arteries. In contrast, the lamellae-like structures were absent from the AAA sample, where the collagen was more randomly distributed, suggesting remodelling had taken place. Unlike the arterial findings however, the SMC content of the AAA IMV did not appear to be markedly reduced compared to the control vessel. This may reflect the much less severe changes that had occurred in this tissue compared to the aneurysmal abdominal aorta.

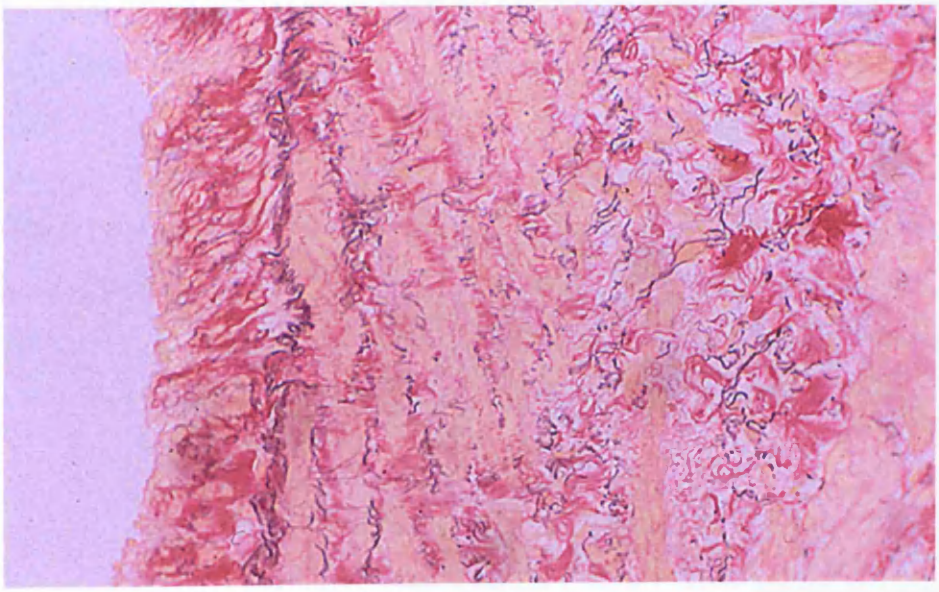
Thus, these findings suggested that, like the aorta, elastolytic processes were occurring within the venous tissue of aneurysm patients. However, it was not clear whether this was a manifestation of local cellular production of proteases, or a result of proteases produced by infiltrating leukocytes. Further studies may answer these questions.

Figure 8.1 **Micrograph (x100) of (a) control and (b) aneurysm-derived, elastin van gieson stained, transverse sections of IMV, illustrating intact and organised elastin fibres in the control tissue, compared to the completely fragmented fibres within the AAA-patient derived vein. Other remodelling is apparent, particularly the presence of disorganised collagen in the AAA sample.**

(a)



(b)



8.4 *MMP And TIMP Production By Inferior Mesenteric Vein*

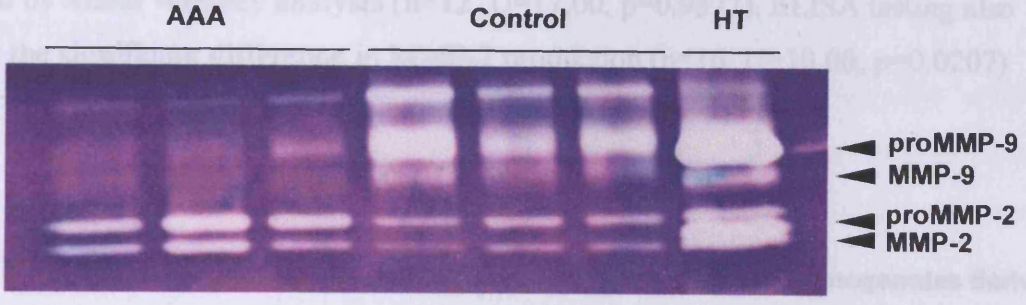
Histological observation suggested that elastolysis was taking place in the aneurysm-derived IMV samples. To confirm the presence of elastolytic activity within the IMV vessel walls, analyses were carried out on whole tissue homogenates to compare the level of production of MMPs between control and AAA tissue samples. Tissue was homogenised in buffer according to its wet weight, and the homogenates dialysed to remove small molecular weight proteins (<10kDa). These homogenates were then subjected to gelatin zymography, western blotting and ELISA. As before, the focus of attention was placed on MMP-2, given its apparent prominence in the aetiology of AAAs as suggested in previous chapters of this thesis.

8.4.1 *Gelatin Zymography Analysis Of IMV Homogenates*

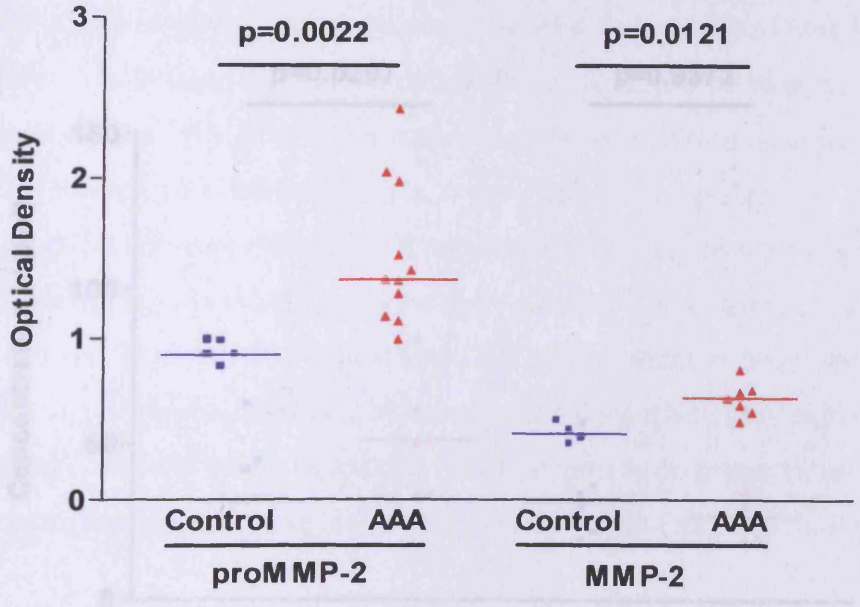
Figure 8.2 shows a typical zymogram obtained from such a comparison. As previously mentioned, this chapter concentrated on the production of MMP-2 in the IMV samples. A prominent difference was seen in the level of proMMP-2 and MMP-2 activity, which was markedly greater in the AAA tissue samples. Scanning densitometry revealed that this difference was significant for both the latent (n=16, U=0.000, p=0.022) and active (n=16, U=1.000, p=0.0121) enzyme, as established by Mann-Whitney analysis. This gelatinolytic activity was consistent with reactivity obtained with an anti-MMP-2 antibody used in western blotting of the homogenates. Activity corresponding to MMP-9 was also present on the zymogram gels in all samples. The levels of this enzyme were much greater in control samples than in those from aneurysm patients (control median 3.489 (3.261-4.118), AAA median 1.184 (0.798-1.655), n=16, U=0.00, p=0.0022), probably due to the nature of the disease that these patients were suffering, which were predominantly ongoing inflammatory conditions of the colon. The most likely source of MMP-9 in these samples was leukocytes, recruited to the colonic mucosa in both colitis and diverticular disease.

Figure 8.2 **(a) Gelatin zymogram of whole tissue homogenates of IMV derived from control and aneurysm patients, illustrating the increased expression of pro and active MMP-2 by aneurysm vein and the presence of large amounts of MMP-9 in control samples. Homogenates were protein equalised before loading. (b) Scattergram of scanning densitometry data for analysis of MMP-2 production by homogenates of inferior mesenteric vein. (c) Summary of scanning densitometry data, expressed as median values with 95% confidence intervals.**

(a)



(b)



(c)

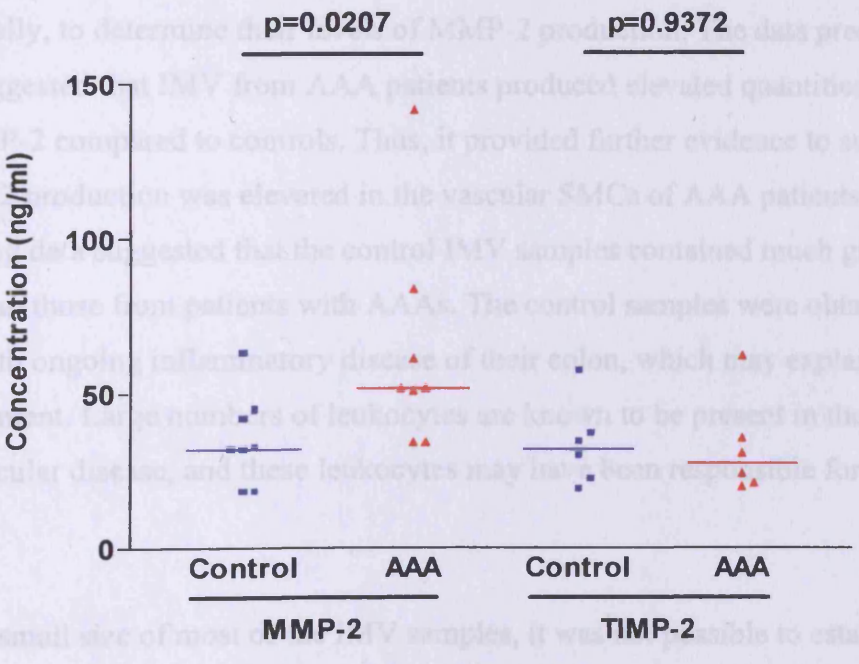
	Control	AAA	p Value
proMMP-2	0.8980 (0.835-1.008)	1.371 (1.215-1.817)	0.0022
MMP-2	0.4115 (0.316-0.517)	0.636 (0.536-0.731)	0.0121

8.4.2 *Western Blotting And ELISA Analysis of IMV Homogenates*

Western blotting with a panel of antibodies failed to detect any MMP activity in the IMV homogenates other than MMP-2 and 9. Western blotting of the homogenates did however demonstrate that TIMP-2 was produced in all samples, but no significant differences in the level of production between samples was revealed. This suggestion was confirmed quantitatively by ELISA analysis of the homogenates, which demonstrated no significant difference in TIMP-2 production between AAA and control patient-derived samples, as determined by Mann Whitney analysis (n=12, U=17.00, p=0.9372). ELISA testing also confirmed the significant difference in MMP-2 production (n=16, U=10.00, p=0.0207) (Figure 8.3).

Figure 8.3 (a) Scattergram of ELISA data obtained from whole tissue homogenates derived from control and aneurysmal IMV. Homogenates were protein equalised before analysis. (b) Summary of ELISA data, expressed as median concentrations with 95% confidence intervals.

(a)



(b)

	Median concentration (ng/ml) with 95% confidence intervals		
	Control	**AAA**	**p Value**
MMP-2	31.70 (22.68-47.37)	51.70 (34.13-93.15)	0.0207
TIMP-2	31.86 (18.70-47.48)	27.35 (15.53-48.86)	0.9372

8.5 *Conclusions*

The experiments conducted in this chapter addressed the hypothesis that elevated MMP-2 expression in aortic SMCs from patients with AAAs may also be detected in other parts of the vasculature. Data from previous chapters had suggested that, whilst this observation was not present in all tissue embryologically derived from mesenchyme, the effect may be specific to the blood vessels, as supported by the study of Ward,[129] which reported systemic dilatation of the vasculature in aneurysm patients.

To test this theory, samples of inferior mesenteric vein were analysed both histologically and biochemically, to determine their levels of MMP-2 production. The data presented in this chapter suggested that IMV from AAA patients produced elevated quantities of latent and active MMP-2 compared to controls. Thus, it provided further evidence to support the theory that MMP-2 production was elevated in the vascular SMCs of AAA patients. However, confounding data suggested that the control IMV samples contained much greater amounts of MMP-9 than those from patients with AAAs. The control samples were obtained from patients with ongoing inflammatory disease of their colon, which may explain their high MMP-9 content. Large numbers of leukocytes are known to be present in the colon in colitis and diverticular disease, and these leukocytes may have been responsible for the MMP-9 detected.

Due to the small size of most of the IMV samples, it was not possible to establish cultured cell lines from the samples used in the present study. To determine the precise role of the SMC in the MMP production in IMV, cell lines must be grown, and subjected to analysis as described in Chapters Five and Seven. The histological revelation of elastin degeneration strongly suggested that the local SMCs were the major source of MMP-2 which was detected on the zymograms. Immunohistochemical studies may also be performed to identify the precise

source of the proteases involved in this ECM degeneration. As in aorta, the most likely sources are the resident SMCs and infiltrating leukocytes. The latter were not visible on histological examination, but may have been present in small numbers.

Thus, the work presented in this chapter provided some evidence to suggest that the SMCs of IMV from patients with AAAs produced elevated levels of proMMP-2 and MMP-2 compared to controls. Further work, to establish the MMP production and expression of isolated cell lines, and to perform immunohistochemical analysis of these tissues, is necessary if firm conclusions are to be drawn on this hypothesis. Control samples obtained from patients without inflammatory disease may also help to remove uncertainty with regard to the source of MMPs in these studies.

CHAPTER NINE

Discussion

9.1 *Review Of Results*

The abdominal aortic aneurysm has been the subject of many scientific and clinical studies, yet its cause remains enigmatic. Some researchers have suggested that AAA is a product of severe atherosclerosis, others suggesting that it arises from a distinct aetiological pathway. It has been unequivocally demonstrated that AAA is associated with an increase in localised MMP production and a dense inflammatory infiltrate. These factors combine to reduce the integrity of the vessel wall and to render it unable to withstand haemodynamic pressures. The present work has presented evidence to suggest that the elevated production of MMP-2 by aortic smooth muscle cells may cause elastolytic degeneration and act as an initiating event in the ECM catabolism and inflammation that signify end-stage AAA disease. Furthermore, this thesis provides some evidence to suggest that the elevated MMP-2 production by the vascular SMCs of aneurysm patients may be a manifestation of a primary genetic change, perhaps in the regulation of MMP-2 gene expression, which is present in the vascular SMCs of susceptible patients.

Initial studies confirmed the MMP production of the medial smooth muscle cells as being limited to MMP-2 and MT1-MMP, plus TIMP-1 and 2. This work confirmed existing data, with the exception of MT1-MMP, which was demonstrated in aortic tissue for the first time. Immunohistochemistry and *in situ* hybridisation studies localised the source of MMP-2 production to the medial smooth muscle cells and to leukocytes dispersed throughout the media and adventitia of AAA tissue. The leukocytes were present in control tissue in small numbers, suggesting that in AAAs they were a secondary phenomenon to aneurysm-initiating, degenerative events, and were unlikely to be the source of proteases which began the process of disease.

The local production of MMP-2 was deemed to be significant, as the complex formed by MMP-2, MT1-MMP and TIMP-2 is capable of elastin degradation. Furthermore, the source of all three, the medial smooth muscle cells, are intimately associated with the majority of elastin found in the aorta. Thus, the capacity for significant elastin damage to be mediated by SMC-derived MMP-2 was clear.

This evidence was augmented by the results of Chapter Five, where isolated smooth muscle cells derived from aneurysm tissue were compared to those from control tissue. However,

difficulty was faced in culturing sufficient numbers of control cell lines, in part due to the scarcity of suitable aorta, but also due to the non-viable nature of much of the tissue obtained. This resulted in low sample numbers for the experiments to follow, and represented the main drawback in studies such as these. The MMP-2 expression of AAA SMCs was found to be significantly elevated, and the MT1-MMP and TIMP-2 expression very similar to controls. These data suggested an imbalance between the MMP and its inhibitor in aneurysm patients, which may result in net proteolysis at the site of production. Attempts to modulate this MMP expression were unsuccessful. Treatment of cells with the inflammatory cytokine IL-1β were found to have no effect on MMP-2 production, and the use of human serum derived from AAA and AOD patients to culture venous SMCs also had no effect on MMP expression. These experiments suggested that the level of MMP-2 production by the AAA SMCs was not being influenced by exogenous stimuli, and may reflect a primary genetic difference between aneurysm-derived and control SMCs.

An interesting phenomenon was the fact that the differences in MMP-2 gene expression illustrated by northern blotting were not reflected in the *in situ* hybridisation studies of Chapter Four. Both techniques directly detected the MMP-2 mRNA transcript. However, histological examination permitted the visualisation of only a small number of cells within the artery wall. Thus, the subtle differences in the level of signal could not be discerned and prevented the use of ISH as a quantitative measure of gene expression. In contrast, northern blotting permitted the comparison of pools of many more cells: the expansion of arterial SMCs in culture effectively amplified the differences in the levels of gene expression. Therefore northerns were used in a semi-quantitative fashion to compare relative amounts of mRNA.

These data prompted the hypothesis that MMP-2 should be able to induce the formation of AAAs in a suitable model. To test this theory, the Wills *in vitro* model of AAA in porcine aorta was modified to include recombinant MMP-2 instead of elastase. The induction of elastin damage and MMP production by endogenous cells of the porcine aortas suggested that MMP-2 was indeed capable of initiating aneurysm like changes in normal aorta. This augmented the theory that overproduction of MMP-2 may be the central, initiating factor in aneurysmal disease, causing elastin damage, which may then bring about secondary effects such as inflammatory cell infiltration and collagenolysis. A further important observation

from this chapter was the point that no collagenolytic MMPs were produced by the cells of the artery wall. This further strengthened the suggestion that collagen degradation was a secondary event, and was mediated by MMPs produced by inflammatory cells, which appeared only after the disease process had begun.

The phenomenon of inflammation in AAAs has been ascribed to the breakdown products of matrix destruction, particularly elastin, which have been reported to be chemotactic for leukocytes. Preliminary data had also suggested that elastin derived peptides (EDPs) may activate MMP-2. In this thesis, EDPs were shown to be able to activate both MMP-2 secreted from cells, and recombinant proMMP-2 *in vitro*. This work suggested that EDPs may act to enhance the destructive environment present within AAAs by activating locally produced MMP-2, and to cause influx of leukocytes.

Changes in the expression of genes may be mirrored in many tissues throughout the body, particularly those derived from the same embryological source. The theory that the differences in MMP-2 expression observed in SMCs may also be present in other mesenchyme-derived tissues was examined in Chapters Seven and Eight. The same tests applied to the aortic SMCs were applied to dermal fibroblast and inferior mesenteric vein homogenates. The data derived from these experiments suggested that MMP-2 expression was similarly elevated in IMV homogenates from AAA patients compared to controls, as analysed by gelatin zymography and ELISA. Histological examination also substantiated the theory that elevated proteolysis was taking place in vessels other than the aorta in patients with AAAs. In contrast, the level of MMP-2 produced by aneurysm-derived and control dermal fibroblasts was not significantly different, suggesting that the changes observed in MMP-2 expression between AAA and control patients may have been confined to the vascular tissue. This hypothesis remains incomplete until further vascular tissue types are tested.

As mentioned, MMP-2 in the aortic wall was predominantly produced by SMCs, and their location as part of the medial elastic lamellae placed them at the centre of speculation as the source of elastolytic activity. This evidence supports that proposed by Patel *et al,*[338] who also reported increased production of MMP-2 by medial SMC cultures derived from aneurysm tissue compared to age matched control cells. However, their work did not examine the role of either MT1-MMP or TIMP-2, nor did it address the levels of expression of any of these genes.

The present thesis thus extends the state of knowledge on the aetiological role of the vascular SMC in AAAs.

A recent study by Davis *et al*[349] suggested that MMP-2 production by mesenchymal cells in the aneurysmal media was particularly prominent when these cells are surrounded by inflammatory macrophages,[349] suggesting highly localised, paracrine modulation of MMP-2 production in AAA. It was suggested by the authors that the macrophages secreted some factor which influenced the release of MMP-2 by medial SMCs. This may occur in a manner not unlike that of tumour cells, which utilise the endogenous MMP-2 production within tissues to degrade basement membrane and metastasise. The secreted MMP-2 is bound at the surface of the tumour cells by MT1-MMP where it is activated, facilitating the degradation of the surrounding matrix and subsequent migration through it. However, the observations of Davis *et al* were not entirely supported by the present work, which did not reveal any instances of increased MMP-2 production in the vicinity of leukocytes in AAA tissue. This may reflect the end-stage nature of the tissue analysed.

9.2 *Proposed Model Of AAA*

The collective data presented in this thesis has allowed the construction of a model of aneurysm formation, as initially proposed in Chapter Five. Strong evidence was obtained to suggest that the vascular SMCs of aneurysm patients produce elevated amounts of MMP-2 compared to control subjects. The presence of increased amounts of MMP-2 in the walls of the blood vessels may lead to greater degeneration of ECM components such as elastin, leading to an inflammatory reaction in response to the liberated breakdown products of matrix proteins.

Two pieces of published evidence support this model. Firstly, the observation by Ward[129] that many arteries in patients with AAAs are also dilated suggests that systemic vascular ECM degradation has occurred. Furthermore, Freestone *et al*[185] demonstrated that MMP-2 was the most prominent MMP in both normal aortas and in small aneurysms, suggesting a fundamental role for MMP-2 in the initiation of the disease process. Moreover, Freestone also showed that MMP-2 prominence diminished as AAA diameter increased, and that MMP-9 content increased. These observations correlated with the reduction in the number of SMCs

and the increase in the density of inflammatory infiltrate. Histological studies revealed the cells of the inflammatory infiltrate to be the major source of MMP-9.

Thus, the data support the theory that inflammation is a secondary phenomenon to a primary degradative event, probably mediated by SMC-derived MMP-2. However, difficulty arises when considering the time-scale over which AAAs appear to develop. If MMP-2 expression were elevated throughout life, an earlier manifestation of ECM degradation might be expected.

Thus, it may be argued that elevated MMP-2 production alone is insufficient for ECM degradation, and that a more significant trauma is required to initiate aneurysm formation. Such criteria may be fulfilled by the damage caused to the arterial wall by the early stages of atherosclerosis, where fatty streak formation, as described in Chapter Two, may provide the circumstances in which the elevated MMP-2 production may become significant. Such a scenario would help to explain the lack of aneurysms in other dilated arteries of patients with AAAs, as described by Ward,[129] as the vessels studied in that report are not associated with atherosclerotic disease. In contrast, the abdominal aorta is a common site for AOD, and indeed, atherosclerotic changes are almost invariably present within AAAs. Also, the morphological characteristics of the abdominal aorta, such as the low number of elastic lamellae, may increase the susceptibility to protease-mediated degeneration.

Therefore, the model proposed above is driven by elastolysis, which is mediated by MMP-2, which is produced by medial SMCs, and possibly initiated by atherosclerosis. This protease driven initiation of AAAs represents one side of a conceptual argument, which is opposed by the inflammation driven initiation of AAA. Perhaps the most interesting version of this concept is that developed by Tilson and colleagues, who propose that AAAs are an autoimmune condition, arising from destruction of self, aorta-specific proteins. This theory demands a reversal of the scheme proposed by this thesis, in that the influx of inflammatory cells would be the initiating event, which would be followed by the profound destruction of ECM proteins seen in mature AAAs. The inflammatory cells, such as macrophages and lymphocytes, produce a range of MMPs, capable of degrading all ECM components as described in Chapter Two. Furthermore, the cytokinetic influence of the inflammatory cells may mediate MMP production by cells in the environment surrounding them, as suggested by the work of Davis *et al.*[349] Like the work presented here, the autoimmunity theory remains

unproven, requiring much further work to identify autoantigens and the mechanisms by which they are targeted.

9.3 *Future Work*

This thesis has presented some evidence to suggest that there may be a difference in the regulation of the MMP-2 gene in patients with AAA. In order to examine this hypothesis more completely, a number of further experiments are necessary. Altered transcription of a gene may occur through a variety of mechanisms. For example, the stability of the mRNA transcript may be greater than is usual, permitting an extended period of translation and hence increased protein synthesis. To examine this an mRNA stability assay would be performed. The longevity of the transcripts in two cell populations is compared by northern blotting of RNA extractions performed over a series of time points after inhibition of transcription. Decay of the mRNA may be assessed by the measurement of the signal as measured by densitometry.

Alternatively, increased detection of a particular transcript may reflect a generalised upregulation of transcription in that cell line. This phenomenon should be rendered insignificant, having established the relative levels of expression of housekeeping genes such as GAPDH or β-actin during the original northern analysis, as was done here. However, to definitively conclude increased transcription of a gene in one cell line versus another, a nuclear run-on assay should be performed. This involves the extraction of nuclei from the cells of interest, permitting the continued extension of nascent mRNAs *in vitro*. The inclusion of radioactively labelled ribonucleotides within the reaction permits the labelling of extending transcripts. Addition of these labelled extracts to immobilised probes for the gene(s)of interest and housekeeping genes, and comparison of relative signals obtained, allows more definitive comparisons of the level of transcription occurring.

Having established that stability is similar and that transcription is indeed elevated for the gene of interest, the regulation of gene expression then needs to be examined. As described in Chapter One, MMP-2 gene expression is not readily modulated, due to the lack of transcription factor binding motifs within its promoter. However, evidence has begun to accumulate that control of MMP-2 transcription is much more complex than first believed. The work done by Lovett and colleagues has demonstrated a number of previously unknown

facets to the regulation of MMP-2 in glomerular mesangial cells. They identified an upstream enhancer element which conferred high level, tissue specific expression of the gene.[350] This enhancer bound the transcription factors YB-1 and AP-2 in a complex which facilitated single-stranded DNA formation and access of polymerase. This motif was analogous to the r2 enhancer identified in HT1080 cells by Frisch *et al.*[351] Furthermore, p53 has also been shown to exert some form of control on MMP-2 transcription, as demonstrated separately by Lovett *et al* (personal communication) and by Bian and Sun.[352]

Transfection studies, analogous to those performed by Harendza *et al,*[353] may determine the presence of transcription factor binding sites within the MMP-2 gene promoter in vascular SMCs, and may permit the localisation of motifs important in the tissue-specific regulation of gene expression. Further studies to characterise the binding protein(s) must then be undertaken, utilising gel shift, DNase footprinting and expression library screening techniques as appropriate. Polymorphism screening may also yield mutational differences in the upstream DNA, although this approach may prove laborious until a specific region of the gene has been identified for closer inspection by the previous methods.

The experiments involving the porcine aortic model of AAA may also be extended in a number of ways. Firstly, the development of the model by Wills *et al* to include autologous macrophages within the culture environment may provide further evidence on the role of the infiltrating cells in AAAs. This may also further define the chronology over which ECM degeneration and inflammation occur, and their relative influence on events. Further modification of this model may involve the use of recombinant TIMP-2, in an attempt to block the degradation of elastin which is characteristic of AAAs. Other MMP inhibitors may also be utilised to determine their ability to block ECM breakdown in AAA disease.

Further clarification of the specific interaction between EDPs and MMP-2 is an important area for future investigation. The data presented in this thesis indicated that EDPs may directly activate proMMP-2 *in vitro*, suggesting an ability to induce pro-peptide cleavage. This surprising finding implies an unknown mechanism for MMP activation, and has no analogue in studies reported to date. Detailed examination of the amino acid sequence of EDPs, and their likely interactions with MMP molecules, may yield possible binding motifs and reveal possible routes to conformational changes.

Finally, the studies on IMV demand further elaboration, to determine both the identity, and source, of elastolytic activity within the venous tissue. Histological studies suggested elastolysis was taking place, and that substantial remodelling of the ECM had occurred in samples derived from patients with AAAs. Immunohistochemical analysis of these samples may reveal the production of elastolytic MMPs, and would determine their source. They would also permit the identification of infiltrating leukocytes, which may profoundly influence ECM homeostasis within the vessel wall.

Furthermore, studies on isolated cell lines from the IMV samples must be undertaken, to establish the possibility that resident SMCs may be producing elevated amounts of MMPs in the manner of aortic SMCs derived from patients with AAAs. The studies on tissue homogenates presented in this thesis begin to answer this question, suggesting elevated production of MMP-2 within the tissue, but these data fail to exclude the potential role of leukocytes in ECM breakdown. Difficulty was presented by the presence of large amounts of MMP-9 in the control group in the present IMV studies. These may be overcome by targeting a source of tissue without inflammatory disease.

9.4 *Closing Comments*

Aortic aneurysm represents a dangerous condition, which will continue to pose a serious risk to many elderly people whilst current methods of detection remain. Present surgical intervention is effective in repairing the dilated artery once detected, but the failure to identify AAAs in many patients restricts the use of this option. The growing evidence, added to by this thesis, of a genetic basis for AAA, suggests that a test for disease susceptibility may be developed. The identification of a genetic basis for AAA may allow screening at an early age, thus pinpointing individuals prior to arterial degeneration.

Pharmacological treatment of those patients with an established aneurysm is limited at present. The MMP inhibitor agents currently undergoing clinical trials appear to induce severe systemic side-effects, and may not represent a good future prospect for diseases involving degradation of the extracellular matrix. Instead, it seems that a more profitable approach to this problem would be to target specific MMPs, perhaps by synthetic inhibitors, or if this induces other problems, to target individual MMP genes. Gene therapy, perhaps involving the localised delivery of antisense DNA, or expression vehicles to produce inhibitory agents, may

be a more precise and elegant solution. Delivery systems and the longevity of this approach remain imperfect, however.

By providing evidence that a tissue specific alteration in MMP-2 gene expression may be responsible for the elastin degradation that initiates aneurysm formation, and the proposal of a novel model of aneurysm initiation, it is hoped that this thesis may contribute to the existing knowledge base on the aetiology of AAAs. Confirmation of this work, and characterisation of putative differences in the vascular tissue-specific control of MMP-2 gene expression in AAA patients, may lead to further avenues of interest in MMP research, and may prove fundamental to the identification and treatment of AAA in years to come.

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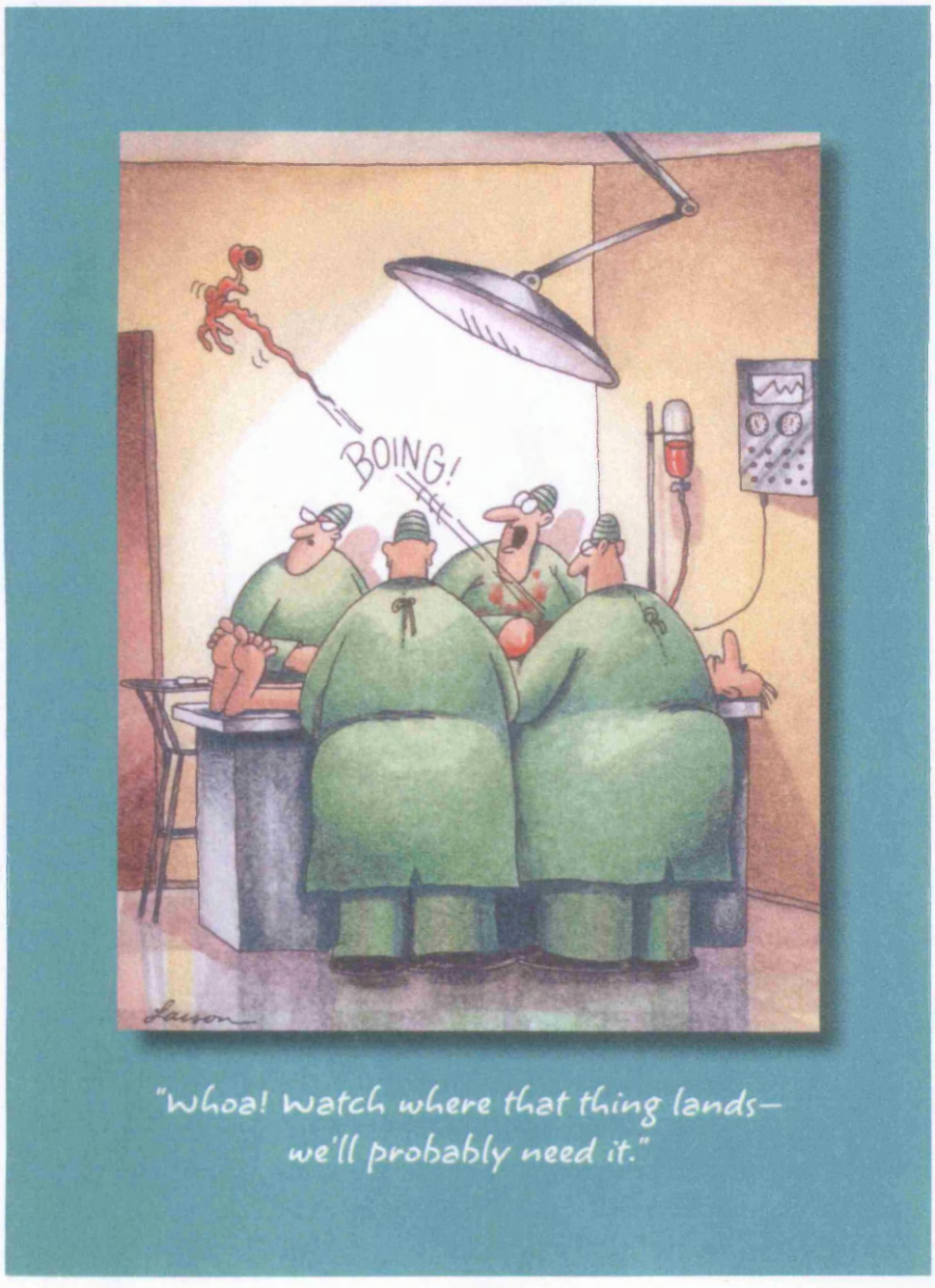
This thesis is dedicated to "The Boys"



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