

The Role of Matrix Metalloproteinases in Carotid Plaque Morphology

Ian Magnus Loftus

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The Department of Surgery

Leicester University

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**Ian Loftus
January 2001**

‘The remedy is worse than the disease’

Francis Bacon

(1561-1626)

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Synopsis

Thromboembolic disease secondary to atherosclerosis is the commonest cause of myocardial infarction and ischaemic stroke. The atherosclerotic plaque develops into a complex structure consisting of a lipid rich core and a connective tissue matrix. Although this causes progressive arterial narrowing, as long as the smooth surface of plaque remains intact it rarely causes significant clinical problems. However if the plaque undergoes acute change such as rupture or ulceration, exposure of highly thrombogenic material in the plaque core leads to thrombus formation with subsequent embolisation or vessel occlusion. There is considerable evidence to support the theory that such acute changes immediately precede the onset of clinical symptoms.

Targeting pharmacotherapy towards preventing acute plaque change could in theory reduce the risk of stroke and myocardial infarction in patients with atherosclerosis. The potential for therapeutic intervention to prevent disease progression is therefore a very attractive option, but the precise cause of acute plaque disruption and thus the target for pharmacotherapy, has been elusive.

There is increasing evidence that a group of proteolytic enzymes called matrix metalloproteinases (MMPs) are intimately involved in the atherosclerotic disease process. The plaque is a dynamic structure, undergoing continuous remodelling of the extracellular matrix upon which its structural integrity depends. MMPs represent the main physiological regulators of the extracellular matrix, and any imbalance between the level of MMPs and their inhibitors could cause increased matrix degradation.

The hypothesis in this study was that a localised imbalance in the level of MMPs and their inhibitors may be associated with plaque instability and the onset of clinical events. The aim of the study was to quantify the major MMP/TIMP subtype levels within carotid plaques and to correlate them with clinical and histological features of plaque instability.

Work from this thesis has been published as follows:

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- Loftus IM, Naylor AR, Goodall S, Crowther M, Jones L, Bell PRF, Thompson MM.
Increased MMP-9 Activity in Unstable Carotid Plaques- A Potential Role in Acute
Plaque Disruption.
Stroke 2000;31:40-47.
- McCarthy MJ, Loftus IM, Thompson MM, Jones L, London NJM, Bell PRF,
Naylor AR, Brindle NPJ. Angiogenesis and the atherosclerotic carotid plaque: an
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- The Royal Society of Medicine, London 1999.
- Surgical Research Society, Brighton 1999 and Cardiff 2000.
- The American Heart Association, New Orleans, 2000

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The Ernest Frizelle Clinical Prize 1998
The Moynihan Prize 1998

CONTENTS

SECTION ONE

INTRODUCTION

- Chapter 1** Atherosclerosis
- Chapter 2** Plaque Instability and Acute Disruption
- Chapter 3** Matrix Metalloproteinases
- Chapter 4** The Role of MMPs in Vascular Disease
- Chapter 5** MMP Inhibition
- Chapter 6** The Management of Carotid Disease

SECTION TWO

METHODS

- Chapter 7** Patients and Methodology

SECTION THREE

RESULTS

- Chapter 8** Histology and MMP Levels Based on Symptom Groups
- Chapter 9** MMP Levels in Association With Other Features of Plaque Instability
- Chapter 10** Localisation of Production and Expression of MMP9 to the Plaque

SECTION FOUR

DISCUSSION AND FUTURE WORK

- Chapter 11** Discussion
- Chapter 12** Future Work

SECTION FIVE

APPENDICES AND REFERENCES

SECTION ONE: INTRODUCTION

CHAPTER ONE

ATHEROSCLEROSIS

1.1 Introduction to Atherosclerosis

Epidemiology

Risk Factors

The Normal Artery

Morphology of Atheroma

1.2 Pathogenesis: The Response to Injury Hypothesis

Introduction

The Response To Injury Hypothesis

The Role of Endothelial Injury

The Role of Macrophages

The Role of Smooth Muscle Cells

The Role of Lipids

The Role of Lipid Trapping and Apoptosis

1.3 Pathogenesis: The Role of Infectious Agents

Introduction

Chlamydia Pneumoniae

Helicobacter Pylori

Cytomegalovirus

Targeting Pharmacotherapy Towards Infectious Agents

1.4 Other Potential Antigens

1.5 Summary

1.1 Introduction to Atherosclerosis

Epidemiology

Atherosclerosis is a chronic disease of the arteries, existing as a named entity for less than 100 years. It is far from a disease exclusively of the twentieth century, arterial changes consistent with atheroma being described in mummies from the Eighteenth Dynasty of the pharaohs of Egypt(Shattock,). While recognised to have a multifactorial aetiology, many aspects of atherogenesis remain unclear.

Atherosclerosis overwhelmingly contributes to more mortality- approximately half or more of all deaths- and serious morbidity in the Western world than any other disorder, particularly in the form of strokes and myocardial infarction (Figure 1.1). Improvements in treatment for patients with both coronary and carotid artery disease plus various preventative measures have reduced the death rates from myocardial infarction and stroke. Between 1963 and 1995 there was a 50% decrease in the death rate from ischaemic heart disease and a 70% decrease in death from stroke in the United States. However, the overall prevalence of the disease remains largely unchanged. In the United Kingdom, arterial deaths account for 37% of all deaths in men under 75 years of age, compared to just over 30% of deaths caused by cancer(British Heart Foundation / Coronary Prevention Group Statistics Database, 1995). This accounts for around 12% of the combined NHS and social services budget(Department of Health, 1998). Considering strokes alone, 0.25% of the population per year suffer a stroke for the first time(Stroke Association, 1996), and strokes account for 6% of all premature deaths, a quarter of survivors being dependant upon carers(Cassidy and Gray, 1991).

Atherosclerosis is most common in North America, Europe and other developed nations, whilst relatively uncommon in South America, Africa and Asia. For example, the mortality rate for ischaemic heart disease in the United States is six times higher than in Japan. However, Japanese residents who move to America acquire the same predisposition as the American population.

Risk Factors

Risk factors which predispose to atherosclerosis have been identified by prospective studies such as the Framingham Study(Truett et al., 1967; Dawber and Kannel, 1966). Age is a dominant influence in the pathogenesis of atherosclerosis and men are more prone than women. Following menopause however, the incidence of ischaemic heart disease in women rises to equal that of men. There is a familial predisposition towards atherosclerosis, largely related to familial clustering of other risk factors, in particular hypertension(Kannel et al., 1969), diabetes and disorders of lipoprotein metabolism(Kannel et al., 1969). Cigarette smoking is an important risk factor for atherosclerosis, a packet per day increasing the risk of ischaemic heart disease by 200%(Kannel et al., 1968).

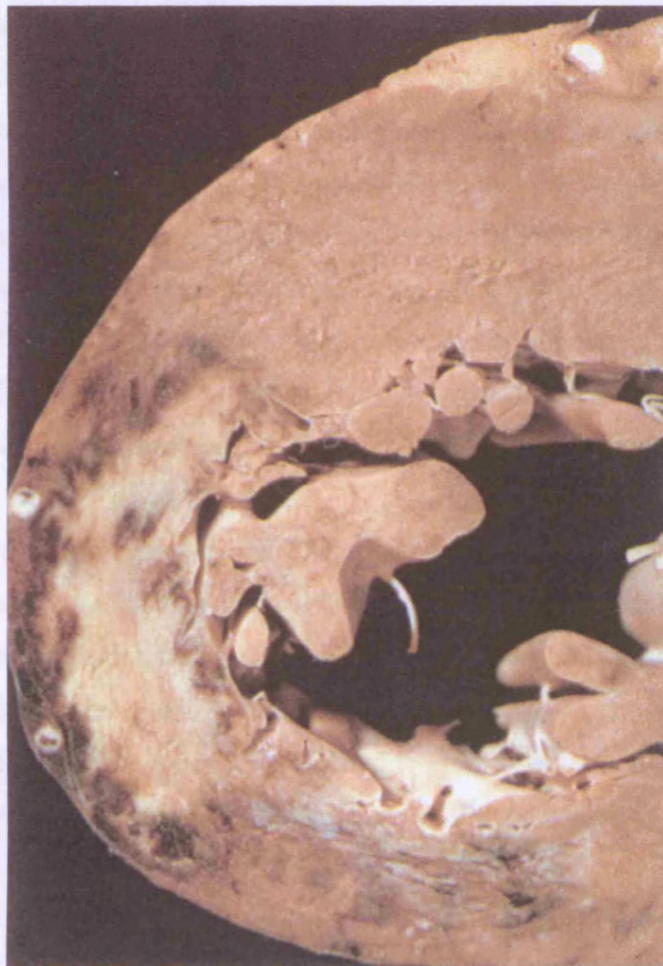


Figure 1.1: *Extensive left ventricular myocardial infarction.*

With regard to hyperlipidaemia, the most important component associated with increased risk is low-density lipoprotein (LDL) cholesterol whilst an inverse relationship exists between the risk of atherosclerosis and the level of high-density lipoprotein (HDL)(Libby et al., 1998). Hypertension becomes more influential with increasing age, particularly in men, with both systolic and diastolic levels exerting an effect(Kannel et al., 1970; Kannel et al., 1969). Elevated plasma homocysteine levels have also been linked to atherosclerosis, probably related to endothelial dysfunction(Bostom and Selhub, 1999). The role of other factors such as viral infections remain the subject of debate.

The Normal Artery

The arterial wall has 3 basic structural constituents, namely endothelium, smooth muscle cells and connective tissue. These are arranged into three concentric layers, an intima, a media and adventitia. In normal arteries, the intima is composed of a layer of endothelial cells overlying minimal connective tissue and separated from the media by the internal elastic lamina. This is a dense elastic membrane interrupted by fenestrae through which smooth muscle cells may migrate. The larger elastic arteries such as the aorta and common carotid artery have a media rich in elastic fibres forming fenestrated layers separated by alternating layers of smooth muscle cells. Smaller branch arteries such as the internal carotid artery and coronary arteries have a media composed predominantly of circularly and spirally arranged smooth muscle cells.

The structural and functional integrity of the endothelium is required for the maintenance of vessel wall homeostasis. This forms a non-thrombogenic blood-tissue interface and has many synthetic and metabolic properties including growth regulation of smooth muscle cells. Endothelial cells may respond to stimuli including immunoregulatory cytokines such as interleukin-1 (IL-1) and interferon-gamma (IF- γ) to promote thrombosis and intimal thickening. Endothelial injury may be responsible, at least in part, for the initiation of atherosclerosis.

Vascular smooth muscle cells synthesise the various components of the extracellular matrix, in particular collagen, elastin and proteoglycans. They are capable of migration and proliferation, an element of the vascular reparative process, but in tandem with an elaboration of the extracellular matrix, represents an important feature of atherosclerosis. Both growth promoters and inhibitors physiologically regulate the migratory and proliferative activity of smooth muscle cells. Promoters include platelet-derived growth factor (PDGF), basic fibroblast

growth factor (bFGF) and IL-1. Inhibitors include nitric oxide, endothelial derived relaxing factor (EDRF), IF- γ and transforming growth factor-beta (TGF- β). Disruption of the physiological balance between inhibition and stimulation will lead to smooth muscle cell growth.

Morphology of Atheroma

Atherosclerosis is characterised by intimal lesions called atheromas or fibro-fatty plaques which protrude into the lumen, weaken the underlying media and undergo a series of complications. Plaques have a focal distribution which is almost certainly governed by haemodynamic factors. Lesions are complex consisting of lipid, most of which is derived from plasma, necrotic connective tissue at the bases and a layer of fibro-muscular tissue forming a 'cap' which separates the plaque constituents from the arterial blood in the lumen.

A morphological spectrum of atherosclerotic lesions is recognised both in humans and in animal models of the disease and the American Heart Association has classified the spectrum of human atherosclerotic lesions into 6 groups (Table 1.1)(Stary, 1992; Stary, 1994).

Nomenclature	Histology	Earliest Onset	Clinical Correlation
Type I (initial lesion)	Isolated macrophage foam cells	From first decade	Clinically silent
Type II (fatty streak)	Mainly intracellular lipid accumulation		
Type III (intermediate)	Small extracellular lipid pools	From third decade	
Type IV (atheroma)	Core of extracellular lipid		Clinically silent or overt
Type V (fibroatheroma)	Lipid core and fibrous cap, or predominantly calcific/fibrous		
Type VI (complicated)	Rupture, ulceration, haemorrhage etc.		

Table 1.1: *American Heart Association classification of human atherosclerotic lesions.*

The earliest recognisable lesion of atherosclerosis is the so-called 'fatty streak', an intimal aggregation of lipid filled foam cells and T lymphocytes. These appear in the aortas of some children under the age of one-year and all children older than ten years, regardless of geography, race, sex or environment (Schwartz et al., 1967). The relationship of fatty streaks to atherosclerotic plaques is complex since they also occur in areas not prone to atheroma, though they are related to the known risk factors for atherosclerosis (Haust, 1971). Coronary fatty streaks develop in adolescence at the same anatomical sites that are later prone to develop plaques and animal studies have shown that fatty streaks can precede the development of intermediate lesions (Faggiotto et al., 1984). Thus, while fatty streaks may precede plaques, not all fatty streaks are destined to become advanced lesions.

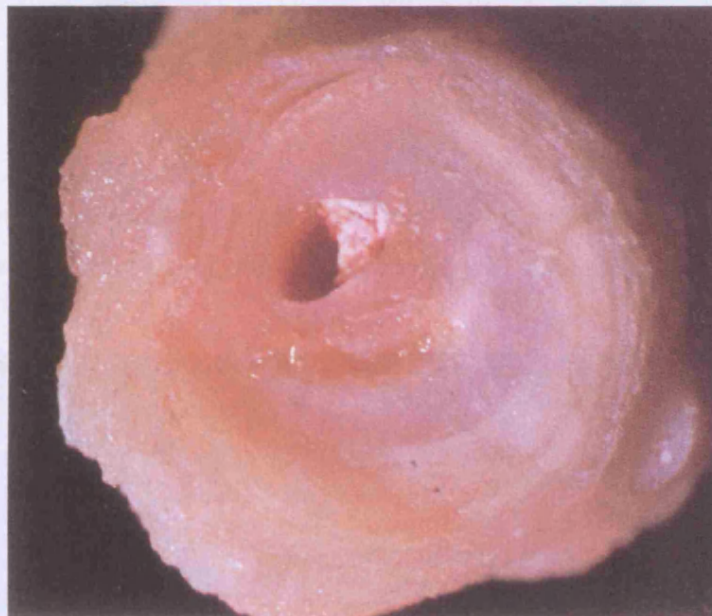


Figure 1.2: *Cross section through a coronary artery showing severe concentric atherosclerosis.*

Intermediate lesions are composed of layers of macrophages and smooth muscle cells with small amounts of extracellular lipid. Gradually these develop an extracellular lipid core and eventually may develop into the more advanced, occlusive lesions of fibrous plaques. The mature plaque consists of a dense cap of fibrous tissue embedded with smooth muscle cells which overlies the lipid rich necrotic core (Figures 1.2-1.4). There is also cellular infiltration, predominantly monocyte-derived macrophages and T lymphocytes, usually concentrated in an area beneath and to the side of the fibrous cap called the shoulder of the plaque (Libby et al., 1996). These components occur in varying proportions, even within advanced lesions, giving rise to a spectrum of lesions.

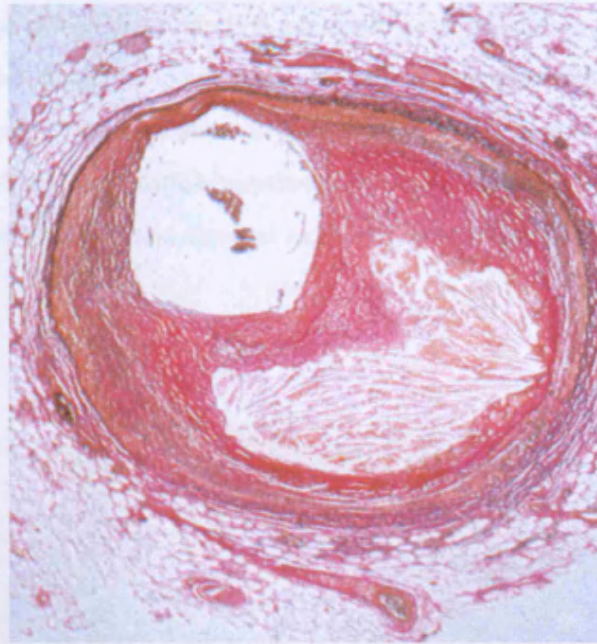


Figure 1.3: Histological section (EVG stain) through severely atherosclerotic coronary artery. Note the lipid rich necrotic core separated from the lumen by an intact fibrous cap.

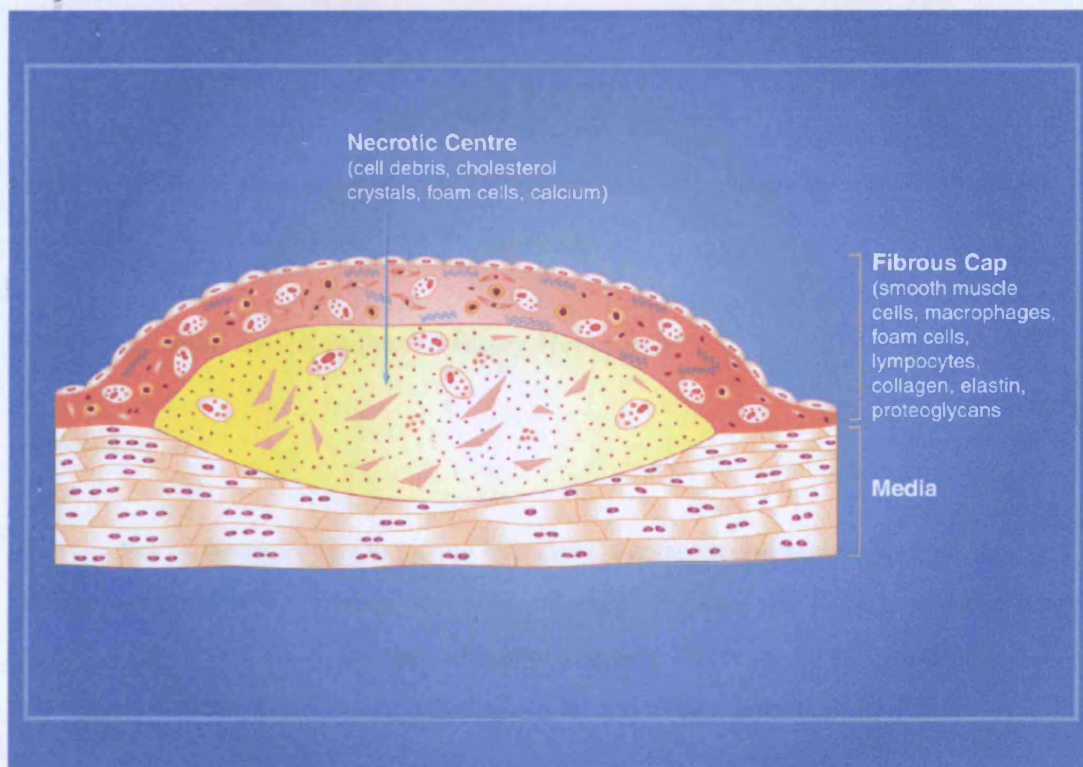


Figure 1.4: Diagrammatic representation of a mature atherosclerotic plaque.

Most sudden deaths from myocardial infarction are due to acute disruption of the coronary plaque, particularly in the shoulder and cap where the inflammatory infiltrate is most intense, leading to thrombosis and occlusion of the artery (Ross, 1999), as shown in Figure 1.5. Acute disruption in the form of plaque rupture, ulceration and haemorrhage also occurs in the carotid artery leading to thromboembolism and cerebral ischaemic events (Carr et al., 1996; Carr et al., 1997).



Figure 1.5: Severe coronary atherosclerosis complicated by plaque rupture and luminal thrombosis, leading to fatal myocardial infarction.

1.2: Pathogenesis: The ‘Response to Injury Hypothesis’

Introduction

The sequence of events leading to plaque formation is complex and probably variable in relation to the degree and duration of exposure to each of clinical risk factors. It is likely that not all plaques arise through the same sequence of events. Theories can be divided into those that suggest that the primary event is some form of endothelial damage or dysfunction, such as the ‘response to injury theory’, and those that propose that smooth muscle cell dysfunction is the primary event.

The 'Response to Injury Hypothesis'

The current consensus is that atherosclerosis occurs as the result of interactions between blood elements, disturbed flow, and vessel wall abnormality, the so-called 'response to injury hypothesis' (Ross, 1993). This theory brings together several observations related to endothelial integrity, macrophage and smooth muscle cell function, lipids and thrombus formation. Central to the theory are the following events, illustrated in Figure 1.6:

- 1) chronic endothelial injury leading to endothelial dysfunction, increased permeability and monocyte adhesion
- 2) adhesion of blood monocytes and leukocytes to the endothelium followed by migration to the intima and transformation to macrophages and foam cells
- 3) lipoprotein insudation and oxidation in the arterial wall
- 4) platelet adhesion to areas of endothelial denudation or to adherence leukocytes
- 5) cytokine and growth factor release from activated platelets, macrophages, endothelial and smooth muscle cells that causes migration of smooth muscle cells from the media intima
- 6) intimal smooth muscle cell proliferation and accumulation of extracellular matrix
- 7) enhanced lipid accumulation, both intra-and extra cellular

The Role Of Endothelial Injury

Endothelial cells are active mediators of many aspects of artery wall function. It is now generally accepted that the initiating event in plaque development is the focal activation of endothelial cells by circulating vasoactive and/or toxic materials, which engender modifications in the endothelial reactivity and metabolism (Ross, 1993). Deleterious agents include endotoxins, hypoxia, products derived from cigarette smoke, specific endothelial toxins such as homocysteine and possibly infectious agents such as Chlamydia. However, other factors must be involved in view of the well-defined tendency for plaque development in specific areas of the arterial tree, namely at the ostia of vessels, branch points, and along the posterior wall of the aorta. The haemodynamic disturbances that occur during normal circulatory function and the adverse effects of hypercholesterolaemia are thought to be of particular importance, inducing endothelial transcription for pro inflammatory cytokines, adhesion molecules and coagulation proteins (Glagov et al., 1995).

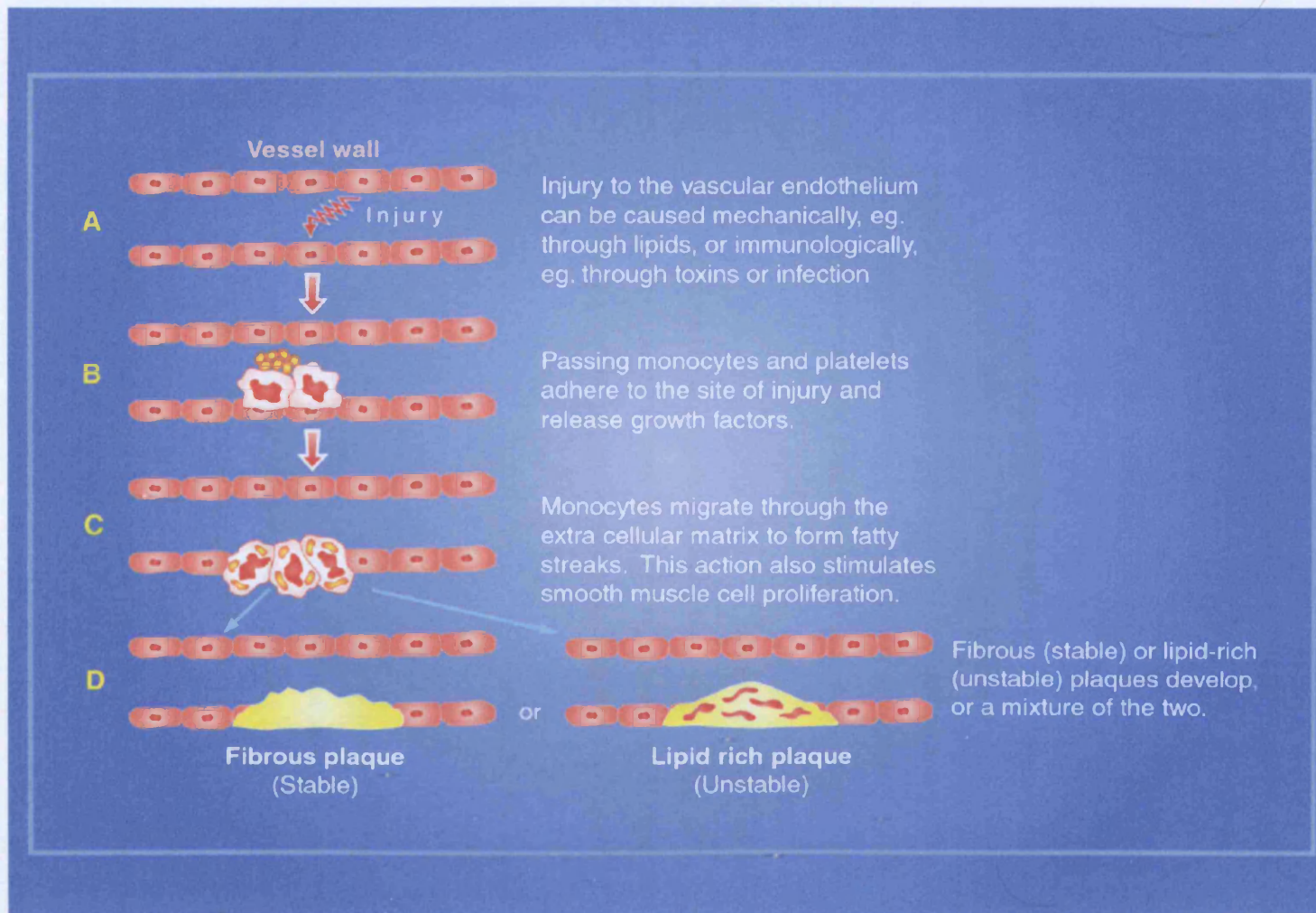


Figure 1.6: The 'response to injury theory of atherogenesis

For example, enhanced expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1)(Raines and Ross, 1995) has been observed. There is an increase in the production of growth regulatory molecules such as PDGF, TNF- α and IL-1(Ross, 1993). These induce smooth muscle cell proliferation and act as chemoattractants, recruiting macrophages and leukocytes into the artery wall and smooth muscle cells into the intima(Ross, 1995). The net result is an increase in endothelial permeability and enhanced monocyte and leukocyte adhesion.

The Role Of Macrophages

Macrophages increase inflammatory cell adhesion and recruitment into the plaque, oxidise low density lipoproteins, ingest lipids to become foam cells, and contribute to smooth muscle cells migration and proliferation. They are also intimately involved in acute disruption of the plaque as described in the following chapter.

Macrophages are intimately involved in the progression of atherosclerotic lesions through interactions with T lymphocytes, smooth muscle cells and endothelial cells. One of the earliest events in atherogenesis is the adherence of circulating monocytes to the endothelium and penetration through tight junctions into the subendothelial space (Figure 1.7). Subsequently, macrophages become involved in atherogenesis through the production of growth agonists including TNF α , PDGF and colony stimulating factors, growth antagonists such as IL-1 and chemoattractants including MCP-1 and TGF- β (Libby et al., 1996).

The effects of macrophage derived cytokines and growth factors are numerous. For example, TNF- α stimulates endothelial cell expression of a recently described receptor for oxidised LDL in early lesions. Oxidised LDL is directly toxic to endothelial cells. In advanced carotid plaques, TNF- α has been shown to stimulate LDL receptor expression in smooth muscle cells and macrophages (Kataoka et al., 1999). Oxidised LDL enhances macrophage recruitment, inhibits migration out of the wall, induces macrophage survival and stimulates proliferation through increased expression of colony stimulating factors (Hamilton et al., 1999).

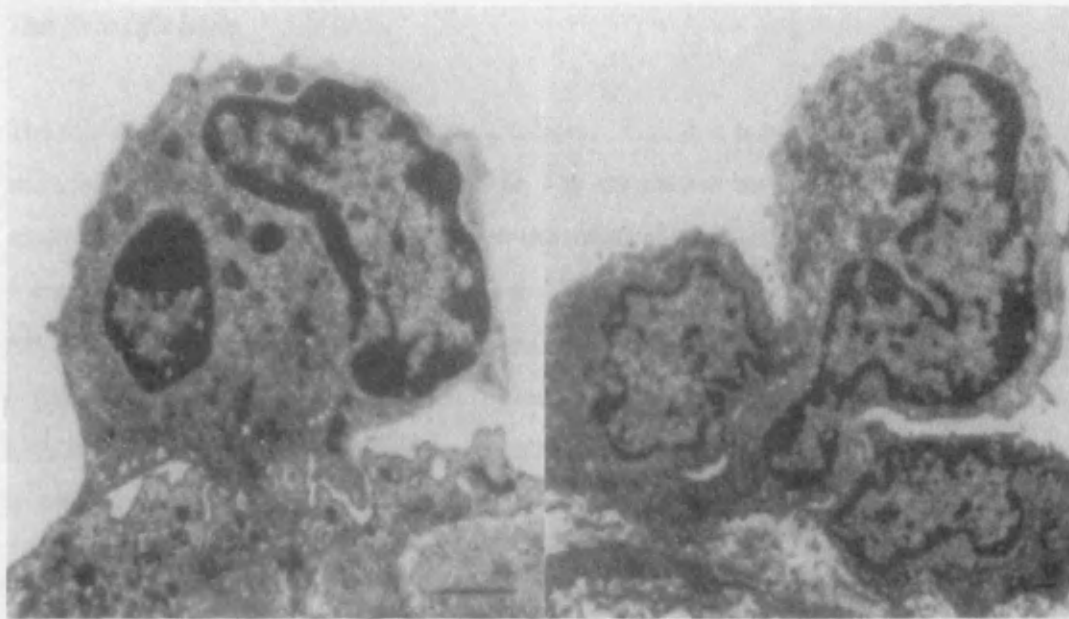


Figure 1.7: Monocyte adherence to the endothelium and penetration between two endothelial cells (from Faggioto, A et al. (1984) *Arteriosclerosis*, 4, 330.)

The Role Of Smooth Muscle Cells

Under the stimulus of chemotactic factors, smooth muscle cells migrate from the media to the intima. Further stimulation, particularly by TGF- β and PDGF, induces cellular proliferation and the production of extracellular matrix. There may be some diversity in that smooth muscle cells from different arteries appear to behave differently, with variable responses to agonists, possibly explaining the propensity for lesions in certain arterial beds (Ross, 1993).

Connective tissue is particularly prominent on the intimal aspect, where it forms the bulk of the fibrous cap of the plaque. Some plaques undergo additional smooth muscle cell proliferation and connective tissue formation to yield fibrous plaques, whilst others retain a central core of lipid laden cells and necrotic debris. The physiological regulation of the extracellular matrix will be discussed in detail later.

The Role Of Lipids

The role of oxidised LDL is probably multifactorial, though it may represent a direct antigenic stimulus of a cell-mediated immune response. The association between LDL oxidation and atherogenesis was first suggested by experiments showing that oxidised LDL causes injury to endothelial cells (Penn and Chisolm, 1994) and further supported by studies showing a reduction in the progression of atherosclerosis with antioxidant therapy (Steinberg, 1995). The expression of a lectin-like receptor for oxidised LDL has recently been described on endothelial cells in human carotid atherosclerotic lesions (Kataoka et al., 1999) and impairment of endothelial function may occur through the production of oxygen free radicals (Feron et al., 1999).

Lipoproteins accumulate within the intima at sites of increased endothelial permeability. Macrophage oxidation by macrophage derived free radicals to derive oxidised LDL, and scavenger receptor mediated uptake of LDL is believed to be one of the key events in plaque development. Readily ingested by macrophages to form foam cells, oxidised LDL is directly chemotactic for further monocytes, increases monocyte adhesion by the induction of endothelial adhesion molecules (Klouché et al., 1999), inhibits further macrophage motility, prolongs macrophage survival and increases DNA synthesis with increased release of growth factors and cytokines (Hamilton et al., 1999). Oxidised LDL may also indirectly influence extracellular matrix remodelling through increased endothelial cell expression of a membrane metalloproteinase (Rajavashisth et al., 1999a).

Oxidised LDL has been shown to increase T cell DNA synthesis, expression of HLA-DR and IL-2 receptors (Frostedgard et al., 1992). This stimulation is dependent upon the presence of macrophages, which in turn are stimulated to produce IL-1 β . This supports the hypothesis that LDL may be responsible for initiating the cell mediated immune response. Oxidised LDL also directly affects the expression of inducible cyclooxygenase in human macrophages, possibly tipping the equilibrium between tissue healing processes and deleterious inflammation, contributing to the progression of atherosclerosis (Eligini et al., 1999).

Further to their role in early atherogenesis, LDL may also have a role in acute plaque disruption. An association has been made between the extent of coronary artery disease and plasma levels of oxidised LDL (Holvoet et al., 1998b). Elevated levels of malondialdehyde-modified LDL are detectable in the plasma of patients following acute myocardial infarction

but not stable angina (Holvoet et al., 1995), correlating with increased levels of inflammatory and ischaemic markers, C-reactive protein and troponin-I respectively. Malondialdehyde-modified LDL is produced in association with platelet activation and linked to ischaemic injury, and has been proposed as a marker of plaque instability (Holvoet et al., 1998a).

The Role of Lipid Trapping and Apoptosis

The lipid core is clinically the most important part of the plaque, since it constitutes the most thrombogenic constituent. Devoid of supporting matrix, the core is avascular, hypocellular and rich in lipids (Lundberg, 1985). The precise pathogenesis of this area remains controversial (Falk et al., 1995). Insudated blood-derived lipid, mainly in the form of LDL, may be trapped within the extracellular space, or lipid may accumulate through foam cell death (Steinberg et al., 1989). The relative contributions played by direct lipid trapping and foam cell apoptosis are unclear, the latter probably being more important (Witztum, 1994). However, early lesions suggest a gradual accumulation in the absence of foam cells, where LDLs bind to glycosaminoglycans, collagen and fibrinogen (Guyton and Klemp, 1994). This requires clarification, since the early lipid-induced changes in the plaque may predispose the plaque to becoming ultimately unstable, as described in the following chapter.

Interest in the hypothesis of a cell-mediated immune response in atherosclerosis has led to interest in other potential antigens, including infectious agents, heat shock proteins and cell surface glycoproteins.

1.3 Pathogenesis: The Role of Infectious Agents

Introduction

Definitive proof of a causal role for infectious agents in atherosclerosis is lacking, but numerous studies have suggested an association between infectious agents and plaque development, particularly *Chlamydia pneumoniae*, *Helicobacter pylori* (H Pylori), cytomegalovirus (CMV) and Herpes viruses (Weck et al., 1997). Certain infectious agents can evoke cellular and molecular changes supportive of a role in atherogenesis (Epstein et al., 1999).

Chlamydia pneumoniae

Chlamydia pneumoniae is a common Gram negative obligatory intracellular bacteria which causes a spectrum of respiratory tract infections. In the first study to reveal an association with atherosclerosis, IgG and IgA antibodies to the organism were found to be raised in 50-60% of men with coronary heart disease compared to 7-12% of an age matched control group (Saikku et al., 1988). A meta-analysis of serological studies seemed to confirm an association between antibody levels and coronary disease (Danesh et al., 1997). A similar association was found between antibody titres and carotid disease (Melnick et al., 1993). Subsequently the organism was detected within coronary plaques (Shor et al., 1992).

Numerous further studies have confirmed the presence of the agent within plaques (Kuo et al., 1993; Casas-Ciria et al., 1996; Jackson et al., 1997b; Kuo et al., 1997), including more recently carotid lesions (Jackson et al., 1997a; Grayston et al., 1995). The organism can be detected in endothelial cells, smooth muscle cells and macrophages within the plaque (Yamashita et al., 1998) and is more commonly found within atherosclerotic tissue than non-diseased tissue from the same patients (Jackson et al., 1997b). A prospective study of patients in the Helsinki Heart Study confirmed that the presence of elevated antibody to *Chlamydia* was a risk factor for the development of coronary heart disease (Saikku et al., 1992). More recently, similar results have been shown for carotid disease with a higher frequency of seropositivity in patients who have suffered stroke or transient ischaemic attack compared to an age matched control group (Cook et al., 1998; Wimmer et al., 1996). The detection of serum antibodies has been linked, in a relatively small prospective study of 152 men with known risk factors for atherosclerosis, to the risk of developing stroke over a 6-7 year follow-up period (Fagerberg et al., 1999). Seropositivity is associated with plasma lipid profiles known to be independent risk factors for atherosclerosis, and also the risk of coronary heart disease in a specific group of patients with familial hypercholesterolaemia (Laurila et al., 1997; Kontula et al., 1999).

Animal models for *Chlamydia* induced atherosclerosis have been developed. Cholesterol-fed rabbits, which are subjected to repeat nasal inoculations of the organism, develop intimal thickening in the thoracic aorta within 3 months (Muhlestein et al., 1998). Furthermore, weekly treatment with azithromycin prevents this accelerated intimal thickening, though it may be that

the antibiotic prevents lesion development by a mechanism unrelated to its anti-Chlamydia effect (Bauriedel et al., 1998).

Work has shown that Chlamydial interaction with monocytes results in upregulation of TNF α and IL-1 β , (Kaukoranta-Tolvanen et al., 1996; Heinemann et al., 1996), both of which are associated with plaque development. Chlamydial production of an antigen, heat shock protein 60, activates human vascular endothelium, smooth muscle cells, and macrophages and regulates TNF α and matrix metalloproteinase expression in macrophages (Kol et al., 1999; Kol et al., 1998). Infection of human monocytes in culture renders the cells capable of inducing a positive lymphocyte proliferative response for up to 7 days suggesting a role in initiating a local immune response in vascular tissue (Airenne et al., 1999). Further, infection of smooth muscle cells and endothelial cells induces expression of tissue factor and plasminogen activator inhibitor 1 (PAI-1), both procoagulant proteins involved in atherosclerosis, suggesting a link with plaque thrombogenicity (Dechend et al., 1999).

There is some doubt about the sensitivity and specificity of the methods employed for Chlamydia detection, particularly immunocytochemistry and polymerase chain reaction because of some conflicting reports (Weiss et al., 1996; Coles et al., 1998). There are also potential confounding factors in studies of seropositivity, including smoking, which has been shown to effect antibody detection (Hahn et al., 1992). A recent large scale prospective study of 15,000 socioeconomically homogenous healthy men in the United States which was controlled for age, smoking, and other cardiovascular risk factors, failed to show any association between Chlamydia seropositivity and the risk of myocardial infarction (Ridker et al., 1999). Similarly, a case-control study of ischaemic cerebral infarctions failed to show any association between the level of Chlamydia antibody and clinical events when comparing 101 case subjects to 201 matched control subjects (Glader et al., 1999). Recent data showed no association between the histological grading of coronary plaque at post-mortem and the direct detection and distribution of Chlamydial DNA (Thomas et al., 1999). More studies are required to determine the involvement of Chlamydia in early lesions and also to more accurately quantify the 'antigen load' in advanced lesions to determine any correlation with disease severity (Taylor-Robinson and Thomas, 1998).

Helicobacter Pylori

H pylori, usually acquired in childhood, has been associated with an increased risk of developing ischaemic heart disease in a number of studies (Ossei-Gerning et al., 1997). (Raso et al.,). More recent studies have, however, cast doubt upon the role of Helicobacter. Malnick failed to detect the organism in carotid plaques (Malnick et al., 1999) while others have failed to show an association between seropositivity and coronary risk (Folsom et al., 1998; Regnstrom et al., ; Wald et al., 1997). McDonagh suggested that the association was, in fact, an association between the infection and age and social class, known risk factors for coronary heart disease (McDonagh et al., 1997).

Cytomegalovirus

CMV is another common human pathogen. The virus remains latent within macrophages for many years after exposure (Coles et al., 1998), has been detected within carotid plaques (Chiu et al., 1997) and shown to induce vessel wall changes similar to atherosclerosis in animal models (Span et al., 1992). It has recently been suggested that infection with CMV may promote smooth muscle cell accumulation within plaques by inhibition of apoptosis (Tanaka et al., 1999). Serological and epidemiological evidence of an association is lacking. One study of 150 individuals with elevated carotid intimal-medial thickness showed a higher mean antibody titre compared to a non-diseased control group (Nieto et al., 1996) whilst another failed to show any association with increased risk of either coronary events or stroke (Fagerberg et al., 1999). The most recent population based case control study failed, after consideration of all potential confounding factors, to show an association between Helicobacter, Chlamydia or CMV, and the risk of coronary events (Danesh et al., 1999).

Targeting pharmacotherapy towards infectious agents

It remains unclear whether the infectious agents discussed above are primary causes of atherosclerosis or a secondary event, particularly as relatively little is known of the mechanism by which such organisms may cause or enhance plaque development (Coles et al., 1998; Kol and Libby, 1998; Saikku, 1998). Their detection in vascular tissue from young subjects suggests the possibility of a primary aetiological role but is not proof of such (Taylor-Robinson

and Thomas, 1998). The presence of microbes within plaques may simply reflect the inflammatory nature of the disease rather than a pathogenic cause (Capron, 1996).

The evidence linking infectious agents with atherosclerosis has led to 3 randomised trials of antibiotic therapy. In one, 220 consecutive male survivors of myocardial infarction were tested for seropositivity to Chlamydia antibodies (Gupta et al., 1997). Those positive were randomised to receive a 3-6 day course of azithromycin or placebo and then observed for further cardiovascular events over the following 6 months. Seropositive subjects receiving no treatment or placebo were found to have a significantly higher risk of further cardiac event than those receiving the antibiotic, or those who were seronegative. However, the numbers were small and the follow-up period short. A further report described a double blind randomised trial of roxithromycin versus control for 30 days in subjects with unstable angina or myocardial infarction (Gurfinkel et al., 1997). This trial observed a significant reduction in recurrent cardiac events in the antibiotic group, though again the numbers were small (2 episode of myocardial infarction and 2 of sudden death in the placebo group versus 0 in the treatment group).

A more recent larger study of 302 seropositive patients treated for a longer period (3 months of azithromycin or placebo) failed to show any difference either in antibody titres or clinical events, though they did show reductions in $\text{TNF}\alpha$, CRP, IL-1 and IL-6 (Anderson et al., 1999). Clearly larger and longer-term studies are required, and 2 such studies are currently underway (Grayston, 1999) (Schussheim and Fuster, 1999). However, as yet the role of infectious agents remains unproved.

1.4 Other Potential Antigens

Other candidate antigens capable of inducing an immune response in the vascular wall have been investigated, including heat shock proteins (hsps). These represent a ubiquitous family of highly conserved proteins, which are increased in a variety of conditions, including inflammatory reactions, functioning to stabilise cellular proteins (Young and Elliot, 1989). Interest in these proteins derived from the observation that rabbits immunised with hsp60/65 developed atherosclerosis (Kleindienst et al., 1995), but only in the presence of other known cardiovascular risk factors such as hypercholesterolaemia (Xu et al., 1996). Further work by the same group revealed that increased expression of hsp65 in rabbits (which has a high degree

of sequence homology to mammalian hsp60) coincided with a population of infiltrating T lymphocytes (Xu et al., 1993). These lymphocytes respond specifically to hsp65 in vitro when isolated from atherosclerotic lesions, more than circulating lymphocytes, suggesting a possible specific role in atherogenesis.

Atheromatous vessels in humans contain endogenous human hsp60 (Kleindienst et al., 1993) which, when expressed by heat-shocked endothelial cells, can provoke an autoimmune reaction mediating endothelial cytotoxicity (Schett et al., 1995). As mentioned above, chlamydial hsp60 co-localises with human hsp60 within plaque macrophages and both can induce macrophage production of pro-atherogenic cytokines such as IL-1, plus MMPs.

Another potential antigen is β_2 -glycoprotein (β_2 GPI), a plasma protein whose role is unclear but is known to be a major target for thrombosis-associated antiphospholipid antibodies. Carotid endarterectomy specimens have recently been shown to contain abundant levels of β_2 GPI associated with endothelial cells and macrophages, and co-localised with T-lymphocytes, suggesting a possible link with an immune-mediated reaction (George et al., 1999). Again, however, further work is required to assess the immunogenicity of β_2 GPI and its role in the complex events surrounding atherogenesis.

1.5 Summary

Atherosclerosis continues to cause considerable morbidity and mortality, particularly in the western world. While risk factors have been clearly identified, their precise roles in early atherogenesis are complex. The early development of the plaque is dependent upon interactions between damaged endothelial cells, vessel wall smooth muscle cells and circulating inflammatory cells by the release of cytokines, growth factors and cell adhesion molecules. Plaque formation may represent a cell-mediated immune phenomenon, with a variety of potential antigenic agents identified. Shear stress and flow considerations also play a part. The final outcome of a plaque depends on whether it becomes unstable, leading to acute disruption of the surface and exposure of the thrombogenic core to the luminal blood flow.

CHAPTER TWO

PLAQUE INSTABILITY AND ACUTE DISRUPTION

2.1 Introduction to Plaque Instability

2.2 Factors Involved in Plaque Disruption

Introduction

The Size and Consistency of the Atheromatous Core

Plaque Cap Thickness and Content

Pulse Pressure and Shear Stress

Inflammation Within the Plaque

Angiogenesis in Plaque Instability

The Role of Apoptosis in Instability

2.3 Plaque Disruption and Thrombogenesis

Introduction

Tissue factor

Other Factors

2.4 Summary

2.1 Introduction to Plaque Instability

It may take decades for atherosclerosis to evolve into the mature plaques responsible for the onset of ischaemic symptoms. Whilst plaque growth due to smooth muscle cell proliferation, matrix synthesis and lipid accumulation may narrow the arterial lumen and ultimately limit blood flow, uncomplicated atherosclerosis is essentially a benign disease. It is now established that acute plaque disruption with superimposed thrombosis is responsible for the onset of clinical ischaemic events (Ravn and Falk, 1999). Plaque disruption involves the breakdown of the endothelium and underlying connective tissue of the cap, exposing core lipid to the blood stream promoting thrombosis and/or embolism (Figure 2.1). The vital question in plaque pathogenesis is why, after years of indolent growth, life-threatening disruption and subsequent thrombosis should suddenly occur. Furthermore, plaque stabilisation may prove to be an important clinical strategy for preventing the development of complications (Shah, 1999; Knight, 1999).



Figure 2.1: *A carotid plaque that has undergone ulceration with subsequent thromboembolism causing transient ischaemic attacks.*

Much of the work performed in this area describes events in coronary atherosclerosis, though it seems likely that this mirrors the process in carotid plaques. Early pathological studies using post-mortem specimens from fatal cases of acute myocardial infarction have revealed that virtually all cases of coronary thrombosis are related to rupture or fissuring of atheromatous plaques (Davies and Thomas, 1985). In cases of sudden death due to ischaemic heart disease, more than 95% show evidence of acute plaque fissuring or rupture (Davies and Thomas, 1984). Falk discovered that the degree of pre-existing luminal narrowing at the site of rupture determined whether rupture caused occlusive thrombosis or just intimal haemorrhage (Falk, 1983).

Early work utilising carotid plaques retrieved at carotid endarterectomy, highlighted the relationship between the presence of thrombus and the clinical status of patients (Harrison and Marshall, 1977; Gunning et al., 1964). This supported the theory that ischaemic attacks resulted from embolism rather than reduction in cerebral blood flow (Ross Russell, 1961).

A number of subsequent studies showed a relationship between the presence of intraplaque haemorrhage and patient symptoms (Imparato et al., 1979). Persson et al found that intraplaque haemorrhage appeared more frequently in symptomatic patients than asymptomatic patients while Lusby suggested a relationship between the onset of neurological symptoms and development of plaque haemorrhage (Persson et al., 1983; Lusby et al., 1982). Intraplaque haemorrhage may occur following cap rupture or disruption. Conversely, neovascularisation identified in unstable plaques may result in plaque haemorrhage, since new vessels are more fragile and prone to haemorrhage (Tenaglia et al., 1998). The rapid expansion in the volume of the lipid core may increase the stress upon a weakened cap predisposing it to acute disruption.

Van Damme and colleagues showed that 53% of complicated carotid plaques (intraplaque haemorrhage, haematoma, thrombus or ulceration) were symptomatic with corresponding neurological deficit, compared to 21% of simple uncomplicated plaques (Van Damme et al., 1994). More recently, work has shown a close association between neurological symptoms and the presence of carotid plaque rupture (Carr et al., 1996).

2.2 Factors Involved in Plaque Disruption

Introduction

A number of intrinsic and extrinsic factors have been identified that determine plaque vulnerability: the size and consistency of the plaque core, the thickness and collagen content of the fibrous cap, and inflammation within the plaque. Further factors such as haemodynamic stress upon the plaque may ultimately contribute to the cap disruption (Richardson et al., 1989).

The Size and Consistency of the Atheromatous Core

The size and consistency of the atheromatous core is variable and critical for the stability of individual lesions (Figure 2.2). Although extremely variable, on average a coronary plaque is predominantly sclerotic with the atheromatous core making up less than 30% of the plaque volume (Kragel et al., 1989). The variability in plaque composition is poorly understood, with no relationship to any of the identified risk factors for atherosclerosis. Gertz and Roberts (Gertz and Roberts, 1990) reported the composition of plaques in 5mm segments from 17 infarct related arteries examined post-mortem and found much larger proportions of disrupted plaques occupied by atheromatous gruel than intact plaques. Davies found a similar relationship in aortic lesions with 10% and 91% of intact and thrombosing plaques respectively exhibiting lipid core of more than 40% of plaque volume (Davies et al., 1993).

Histological data regarding the necrotic core of carotid vessels is limited though there is considerable evidence to link echolucent plaques on ultrasound, deemed to contain more soft or amorphous tissue, with symptomatology (Reilly et al., 1983; El-Barghouti et al., 1996). Feeley and colleagues demonstrated a significant increase in the proportion of carotid plaques occupied by amorphous material in symptomatic plaques than asymptomatic plaques, though other studies have failed to show such a relationship (Carr et al., 1996).

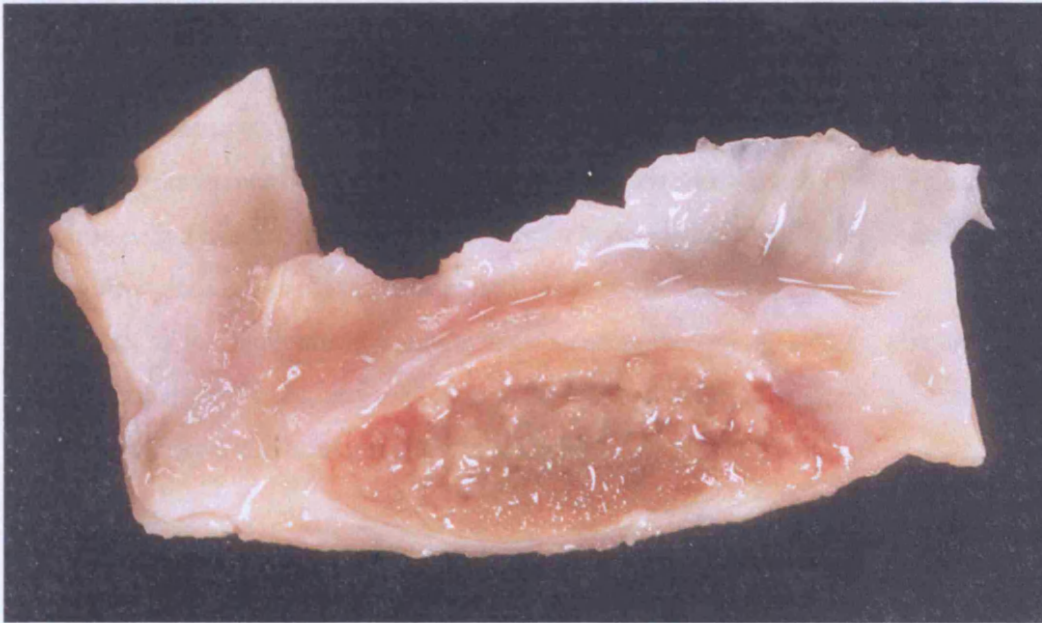


Figure 2.2: Photograph of a mature carotid plaque retrieved from endarterectomy. The lipid rich necrotic core can be clearly seen, separated from the lumen by an intact fibrous cap.

Plaque Cap Thickness And Content

The thickness and collagen content of the plaque cap are important for stability of the plaque (Loree et al., 1992). The most vulnerable area of the plaque is the shoulder region, where the cap is often at its thinnest (Falk et al., 1995). Collagen is vital to the strength of the cap and studies have shown a reduction in the collagen content of the cap around areas of plaque disruption, plus steep transverse gradients of connective tissue constituents across ulcerated plaques (Burleigh et al., 1992). This may result from a reduction in matrix production by smooth muscle cells, also diminished in areas of plaque disruption (Davies et al., 1993), by increased degradation of matrix by proteolytic enzymes, or a combination of excessive matrix degradation and reduced production. A reduction in smooth muscle cells within the fibrous cap would certainly undermine its strength (Davies, 1996). Recently there has been interest in the role of smooth muscle cell apoptosis in plaque cap weakening, caused by a combination of intrinsic and extrinsic factors, particularly macrophage and lipid derived products (Kockx et al., 1998; Kockx, 1999).

Pulse Pressure and Shear Stress

According to Laplace's law, the higher the blood pressure and the larger the luminal diameter, the more circumferential tension develops in the wall (Lee and Kamm, 1994). This phenomenon combined with a radial compression of the vessel wall may lead to unbearable stress in vulnerable regions of the plaque, particularly the cap and shoulder (Cheng et al., 1993). For fibrous caps of the same tensile strength, caps covering moderately stenotic plaques are probably more prone to rupture than caps covering severely stenotic plaques, because the former have to bear a greater circumferential tension (Loree et al., 1992).

The propagating pulse wave causes cyclic changes in lumen size and shape with deformation and bending of plaques, particularly those with a large soft plaque core. Eccentric plaques typically bend at the junction between the relatively stiff plaque and the compliant vessel wall (MacIsaac et al., 1993). This is accentuated by changes in vascular tone.

High blood velocity within stenotic lesions may shear the endothelium away, but whether high wall stress alone may disrupt a stenotic plaque is questionable (Falk et al., 1995). The absolute stresses induced by wall shear are usually much smaller than the mechanical stresses imposed by blood and pulse pressure (Gronholdt et al., 1998).

Inflammation within the Plaque

Distinct from its involvement in the early pathogenesis of atherosclerosis, inflammation also plays a key role in the destabilisation of the plaque, causing disruption and the onset of clinical ischaemic events (Van Der Wal AC et al., 1997) (Buja and Willerson, 1994). Richardson described the presence of foam cell infiltration at the site of all plaque disruptions in acute coronary thromboses (Richardson et al., 1989). Further, the site of coronary plaque intimal rupture or erosion is characterised by an intense inflammatory cell infiltration, predominantly macrophages and foam cells, but also T lymphocytes and other cells (Van der Wal et al., 1994).

Moreno quantified intense macrophage infiltration in 14% of plaque tissue from patients with unstable angina or recent myocardial infarction, compared to 3% in patients with stable angina (Moreno et al., 1994). These cells demonstrated evidence of recent activation by abundant HLA-DR (Class II) expression and an increase in the percentage of IL-2 receptor positive T-

cells (expressed transiently soon after activation) (Van der Wal et al., 1994; Van der Wal et al., 1998). It has been hypothesised that activated T-cells are involved in stimulating macrophage production of proteolytic enzymes and plasminogen activators, promoting thrombin generation.

Prospective epidemiological studies have shown a clear association between the progression of coronary atherosclerosis and systemic markers of inflammation including white cell count (Ernst et al., 1987) and more recently C-reactive protein (Koenig et al., 1999) and secretory type II phospholipase A₂ (Kugiyama et al., 1999). Until recently it was unclear whether the pattern and magnitude of increased plasma concentrations of inflammatory markers varied with the site and extent of disease. In a detailed study comparing the levels of a number of inflammatory markers amongst patients undergoing coronary arteriography, Erren et al found strong univariate associations between the plasma levels of CRP, serum amyloid A, IL-6, TGF- β , neopterin and procalcitonin and the degree of atherosclerosis. Interestingly however, these associations were lost in multivariate analysis once age, sex and high density lipoprotein cholesterol or fibrinogen were taken into account (Erren et al., 1999). They concluded that, although these markers represent a feature of advanced atherosclerosis, they do not provide discriminatory diagnostic power over and above established risk factors.

Work on carotid plaques is more limited but mirrors the events in coronary and aortic tissue (Figure 2.3). Plaques with evidence of rupture show macrophage and T-lymphocyte infiltration in the cap, shoulder and base (Milei et al., 1998). Jander recently quantified this infiltration in 37 consecutive carotid plaques removed at endarterectomy and described a significant increase in the macrophage and T-cell rich areas in symptomatic compared to asymptomatic plaques, and also in those plaques with evidence of cerebral embolisation detected by transcranial Doppler ultrasound (Jander et al., 1998).

The mediators of communication between the various cell types involved are poorly understood though there has been recent interest in the role of the CD40 immune mediator and its ligand CD40L. CD40L positive T-cells have been identified in human plaques and in vitro, CD40/CD40L interaction induces the production of pro-inflammatory cytokines, adhesion molecules, proteolytic enzymes and tissue factor (Schonbeck et al., 1997; Mach et al., 1997; Karmann et al., 1995). More recently blocking this interaction has been shown to limit atherosclerosis in vivo (Mach et al., 1998).

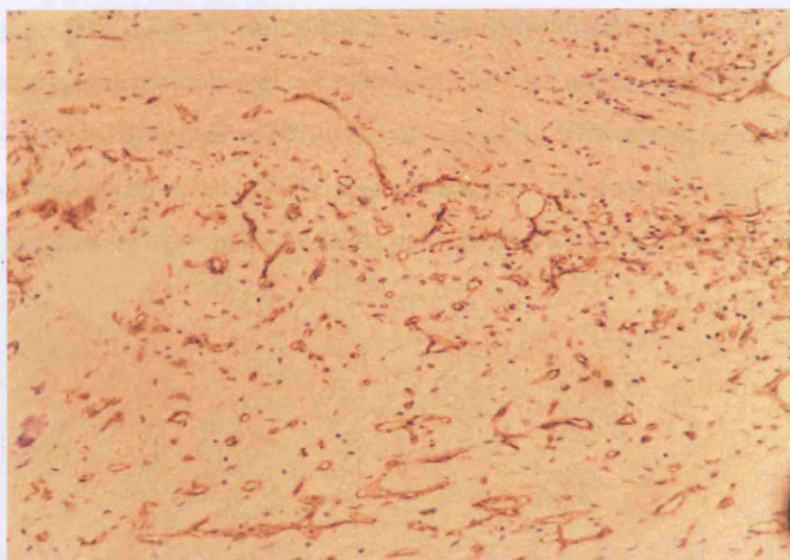


Figure 2.3: *Histological section revealing intense macrophage infiltration in a carotid plaque (x50 magnification, stained with anti-human CD-68 antibody).*

Both intact and disrupted plaques exhibit inflammatory infiltration and it could be argued that inflammation is a constitutive part of the plaque. However, evidence of recent and transient activation in unstable plaques supports a theory of direct involvement in disruption. Lendon and co-workers performed mechanical testing of aortic plaques and revealed that foam cell infiltration led to a reduction in tensile strength (Lendon et al., 1991). Further work described the detection of IL-2 receptors on T-lymphocytes, evidence of recent antigenic stimulation, and the presence of HLA-DR positive macrophages capable of antigen presentation, indicative of a cell-mediated immune response within plaques (Van Der Wal AC et al., 1997) (De et al., 1997). This has been supported by evidence in carotid plaques of a dominance of pro-inflammatory and macrophage stimulating cytokines (IL-2 and IF γ) in the most advanced, unstable lesions (Frostegard et al., 1999).

Angiogenesis in Plaque Instability

Angiogenesis, whilst part of normal growth and development, is also recognised in disease states involving tissue remodelling such as malignancy (Eisenstein, 1991). Neovascularisation has been identified in plaques (Barger et al., 1984) (Figure 2.4) and postulated to play a role in atherosclerosis through the provision of growth factors and cytokines to regions of plaque

growth. Angiogenesis may also play a role in plaque de-stabilisation, possibly through promoting intraplaque haemorrhage. One study of coronary arterial specimens obtained at atherectomy revealed the presence of neovascularisation in 50% of specimens from patients with unstable angina compared to 10% of specimens from patients with stable angina (Tenaglia et al., 1998) suggesting a possible role in the pathogenesis of unstable angina.

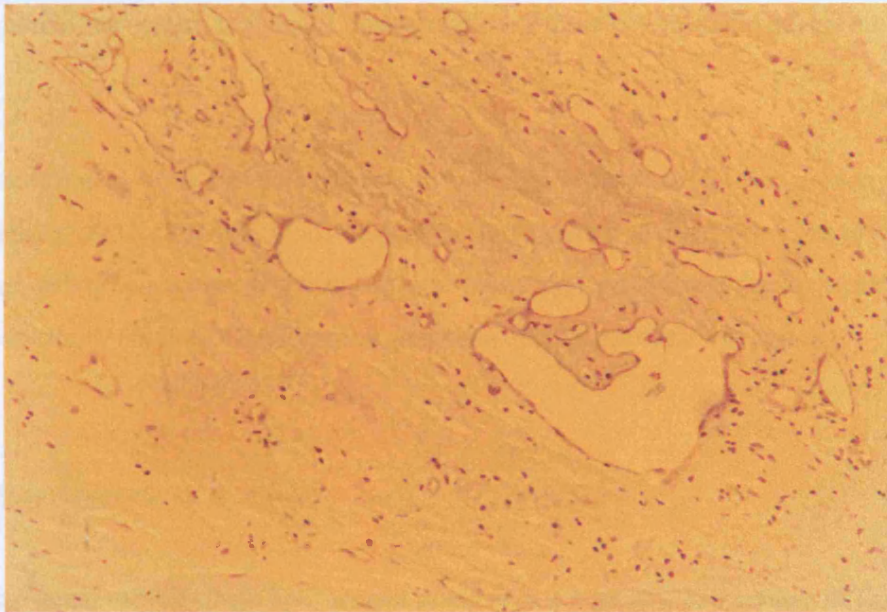


Figure 2.4: Evidence of angiogenesis within a mature carotid plaque (x50 magnification, immunocytochemistry stained with anti-human CD-34 endothelial cell specific antibody).

A further role for angiogenesis may be the recruitment of inflammatory cells into vulnerable areas of the plaque. A recent histological study of plaques showed a significant increase in microvessel density in lipid rich compared to fibrous plaques (De et al., 1999). Perhaps more importantly, most of these vessels were located in the vulnerable shoulder area of the plaque. Immunostaining for inflammatory cells showed a close association between angiogenesis and inflammatory infiltration. Similar work has demonstrated a parallel increase in the expression of leukocyte adhesion molecules in the same vulnerable areas (de Boer et al., 1999).

Angiogenesis involves interactions between endothelial cells and adjacent cells with components of the basement membrane matrix. MMP activity is required for such interactions, probably especially MMP-2 and MT1-MMP (Haas and Madri, 1999). TIMPs have been shown

to prevent angiogenesis and conversely inhibition of TIMPs using cathepsin-B up-regulates MMP activity and stimulates increased angiogenesis(Kostoulas et al., 1999).

In a mouse model of atherosclerosis, angiogenesis inhibitors have been shown to reduce neovascularisation within aortic intima in association with a reduction in plaque growth (Moulton et al., 1999). The mechanism of this is unclear since treatment failed to affect the smooth muscle cell content of plaques. Extrapolation of these results to the human situation is difficult.

Whilst neovascularisation may promote and sustain inflammatory infiltration predisposing these plaque areas to de-stabilisation, the converse may be true, whereby changes in the plaque associated with inflammation may themselves promote angiogenesis and further work is required to clarify the role of angiogenesis in plaque development and disruption.

The Role of Apoptosis in Instability

Unstable plaques exhibit a high lipid content along with a relatively low content of smooth muscle cells, and interest has been raised into the role of cellular apoptosis in plaque instability (Bennett, 1999). A recent, though small, study demonstrated that the proportion of smooth muscle cells undergoing apoptosis and the frequency of cytoplasmic remnants of apoptotic cells were significantly increased in unstable versus stable angina atherectomy specimens (Bauriedel et al., 1999).

Apoptosis of smooth muscle cells and macrophages has been identified within plaques, but only advanced disease with dense macrophage infiltration. Distinct from necrosis, apoptotic cells are deemed to become susceptible to a special form of cell death characterised by a series of morphological changes, starting with shrinkage of the cell membrane to condensation of nuclear chromatin, cellular fragmentation and eventually engulfment of apoptotic bodies by surrounding cells (Kockx, 1999). The presence of pro-apoptotic proteins in advanced plaques, plus the observation that cells derived from plaques but not the media die when brought into culture, suggests that certain cells are programmed to die, with additional factors initiating the apoptotic process (Kockx et al., 1998; Bennett et al., 1995).

Intimal cell apoptosis may account for the low density of smooth muscle cells in unstable plaques, and may contribute to the events leading up to plaque disruption. However, as with neovascularisation, the converse relationship may also be true, and the precise role of cellular apoptosis remains unclear.

2.3 Plaque Disruption and Thrombogenesis

Introduction

Disruption alone would not precipitate ischaemic syndromes without thrombus formation on the plaque surface, so plaque instability and thrombogenicity in tandem predispose to acute clinical events. Platelet adherence to the sub-endothelium after surface disruption leads to activation, with ADP and serotonin release stimulating further platelet recruitment and activation. The importance of platelet activation in acute cardiovascular events is supported by the clear benefit of aspirin in patients with atherosclerosis (Antiplatelet Trialists Collaboration, 1988).

Tissue factor

Much of the thrombogenicity associated with disrupted plaques is associated with the expression of pro-coagulant 'tissue factor' (Libby et al., 1998). In a direct comparison of the level of tissue factor in coronary plaques from patients with stable and unstable angina, the level in unstable plaques was more than two-fold that of stable plaques (Moreno et al., 1996). Positive immunostaining correlated with areas of intense macrophage infiltration and smooth muscle cells, suggesting a cell-mediated increased thrombogenicity in unstable plaques. The increase in tissue factor levels seems to be linked to expression of CD-40 on the macrophage cell surface, the ligand of which is expressed on activated T-lymphocytes among other cells, within atheroma (Mach et al., 1998).

Other Factors

Other factors seem to be involved and the role of platelets is likely to be multifactorial. For example, platelet derived nitric oxide (NO) has been shown to inhibit platelet recruitment and there is evidence of impaired NO production by platelets in patients with acute coronary syndromes(Freedman et al., 1998). Fibrinogen is an important co-factor in platelet aggregation, where activated platelets bind fibrinogen which then binds to glycoprotein receptors on adjacent platelets, stimulating further aggregation. The concentration of fibrinogen rises in acute inflammatory conditions(Coles et al., 1998). Seasonal variation in circulating fibrinogen levels coincide with fluctuations in cardiovascular mortality, and elevated levels have been associated with increased cardiovascular risk(Ernst, 1993). This is related to increased platelet aggregation but also blood viscosity, vascular permeability and leukocyte chemotaxis which are regulated by the end-products of fibrinogen degradation(Murphy et al., 1991).

Von Willebrand factor is also important in platelet adherence and activation, along with P-selectin and platelet surface glycoprotein receptors(Prentice, 1999). Plasminogen activator inhibitor (PAI-1) is the major inhibitor of the fibrinolytic system, and elevated plasma levels of both von Willebrand factor and PAI-1 are associated with subsequent development of a myocardial infarction(Thogersen et al., 1998).

2.4 Summary

Acute plaque disruption precedes the onset of clinical ischaemic syndromes. Exposure of the highly thrombogenic core to luminal blood leads to platelet adherence and thrombosis. Inflammation, and particularly the macrophage, is clearly involved in the process of plaque development and acute disruption as shown in Figure 2.5, though the precise mechanism by which the inflammatory process is initiated remains unclear. The roles of angiogenesis and cellular apoptosis also require further clarification. Unstable plaques have a high lipid content and thin fibrous cap with reduced collagen content and a major component of plaque destabilisation seems to be increased matrix degradation, the primary regulators of which are the matrix metalloproteinases and their inhibitors.

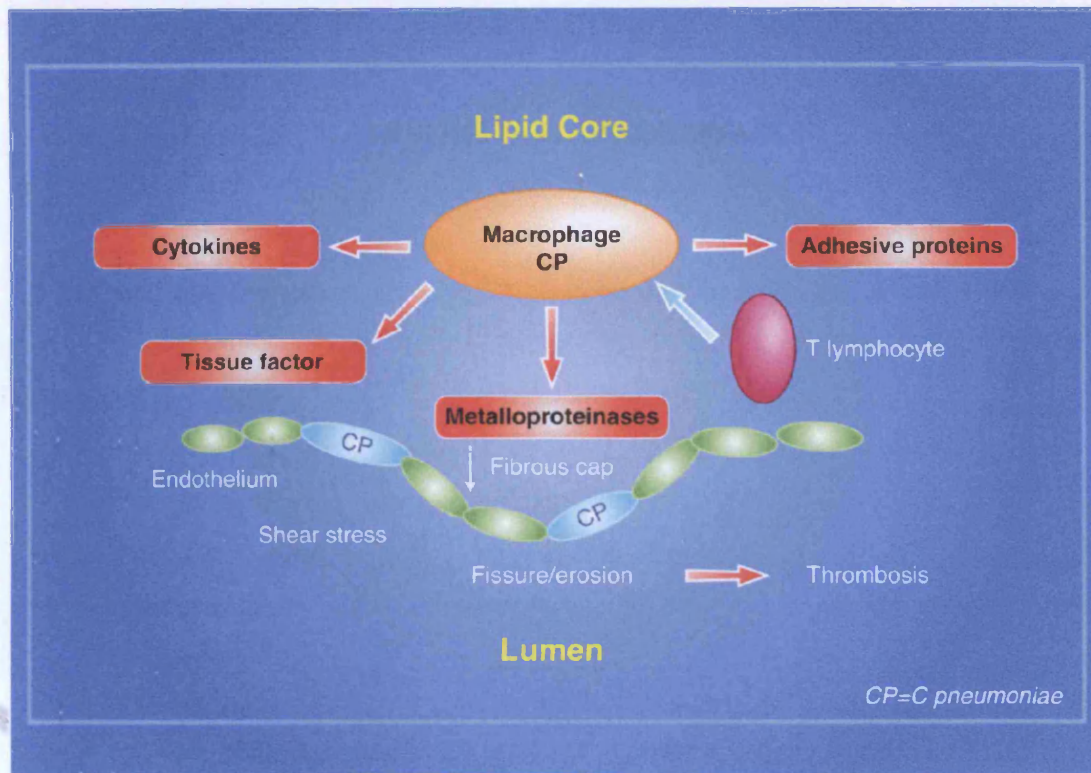


Figure 2.5: Proposed role for the macrophage, which is central to the development and disruption of the mature atherosclerotic plaque.

CHAPTER THREE

MATRIX METALLOPROTEINASES

3.1 Introduction to MMPs

3.2 The MMP Family

Characteristics of MMPs

MMP Subclasses

MMP Activity

Structure of MMPs

MMP Genes

Site of MMP Production

3.3 Regulation of MMP activity

Introduction

Control of Gene Expression

Extracellular Activation of the Pro-Enzyme

MMP Inhibition

3.4 Summary

3.1 Introduction to MMPs

Connective tissue integrity depends upon a balance between resorption and repair of the extracellular matrix (ECM). In terms of degradation and therefore resorption, the activity of proteolytic enzymes is the rate limiting step (Murphy and Docherty, 1992). It is now well established that, among numerous potential proteinases, the MMPs play the major role in the degradation of collagen and other ECM macromolecules.

MMPs are essential in normal healthy individuals, playing a key role in processes such as wound healing (Wysocki et al., 1993; Agren et al., 1998), pregnancy and parturition (Polette et al., 1994), bone resorption (Delaissie et al., 1988) and mammary involution (Talhok et al., 1992). However there is growing interest in a role in disease states where ECM breakdown plays a predominant role (Krane, 1994).

Early interest focused on a pathological role for MMPs in the resorption of periodontal structures in periodontal disease (Page, 1991), the destruction of joints in rheumatoid arthritis (Harris, 1990), and the local invasive behaviour of malignancies (Parsons et al., 1997). It is well recognised that MMP genes are abundantly expressed in these inflammatory and malignant disease states and, more recently, interest has developed in the potential role of MMPs and other proteolytic enzymes in vascular diseases.

3.2 The MMP Family

Characteristics of MMPs

The MMPs are members of a large subfamily of proteinases characterised by tightly bound zinc ions. There are at more than 20 members of the family, which exhibit structural homology, but each of which is the product of a different gene. All members of the MMP family share certain features:

- 1) degradation of one or more components of ECM
- 2) tight binding to zinc ions at their active site
- 3) secretion in a latent pro-enzyme form which requires activation for proteolytic activity
- 4) inhibition by tissue inhibitors of metalloproteinases (TIMPs)
- 5) common amino acid sequences.

MMP Subclasses

The MMPs have been subdivided into 4 major subclasses: collagenases (MMP-1, MMP-8, MMP-13, MMP-18), gelatinases (MMP-2 and MMP-9), stromelysins (MMPs 3,10,11,19 and 20) and membrane type MMPs or MT-MMPs (MMP-14, MMP-15, MMP-16 and MMP-17). The early classification of MMPs was largely based upon substrate specificity as shown in Table 3.1. Initially satisfactory for the collagenases, thought to have a unique ability to degrade collagens of type I, II and III, this classification is now less useful, since many of the enzymes have broad and overlapping substrate specificities.

MMP Activity

The collagenases predominantly digest collagen types I, II and III. Cleavage takes place at a single locus in the component α -chains by scission of the Gly₇₇₅-Ile₇₇₆ or the Gly₇₇₅-Leu₇₇₆ bonds. These sequences do not however dictate enzyme specificity since similar bonds exist in the triple helices of interstitial collagens that are unaffected (Fields et al., 1987). Cleavage is thought to occur at these sites because they occupy a locus that readily unfolds the triple helical structure (Birkedal-Hansen et al., 1993).

Gelatinases digest type IV and type V collagen, denatured collagen and arterial elastin, proteoglycan core protein and some glycosaminoglycans. Within denatured collagens, cleavage has been found to be associated with Gly-Val, Gly-Leu, Gly-Asn and Gly-Ser peptide bonds (Seltzer et al., 1990). The stromelysins demonstrate proteolytic activity against proteoglycan ground substance, but also activate other MMPs. Membrane type matrix metalloproteinases (MT-MMPs) have unique hydrophobic trans-membrane domains and are limited to the cell surface where they function as physiological activators of MMP-2 (Sato et al., 1994).

Structure of MMPs

Evolution of the MMP family from a single prototype has led to conservation of the same basic five domain structure (Figure 3.1), with 70% similarity between members of the same group and 50% similarity between members of different groups (Ye et al., 1998). The first (signal) domain is 17-29 residues long, rich in hydrophobic amino acids, and is essential for secretion

of the peptide from the cell. Once secreted the zymogen loses this domain. The pro-peptide domain is 77-87 residues long and forms the N-terminal domain of the secreted form of the enzyme. This incorporates the 'cysteine switch' mechanism, highly conserved throughout the MMP family, which is essential for maintenance of the zymogen form (Springman et al., 1990). The pro-peptide functions to maintain latency by co-ordination of Cys in the conserved sequence PRCGVPDG to the active centre zinc atom (Woessner, 1994).

The catalytic domain is critically dependent upon the sequence HE.GH.G.HS which forms the putative tridentate zinc binding site (Woessner, 1994). The 3 histidine residues associate with the catalytic zinc ion (Springman et al., 1990). In the active form of the enzyme, a water molecule represents the fourth ligand and is essential for catalysis. The gelatinases MMP-2 and MMP-9 have an additional 3 tandem repeats, homologous to fibronectin type II molecules, inserted in the catalytic domain which specify gelatinolytic activity by facilitating binding to their substrate (Goldberg et al., 1989).

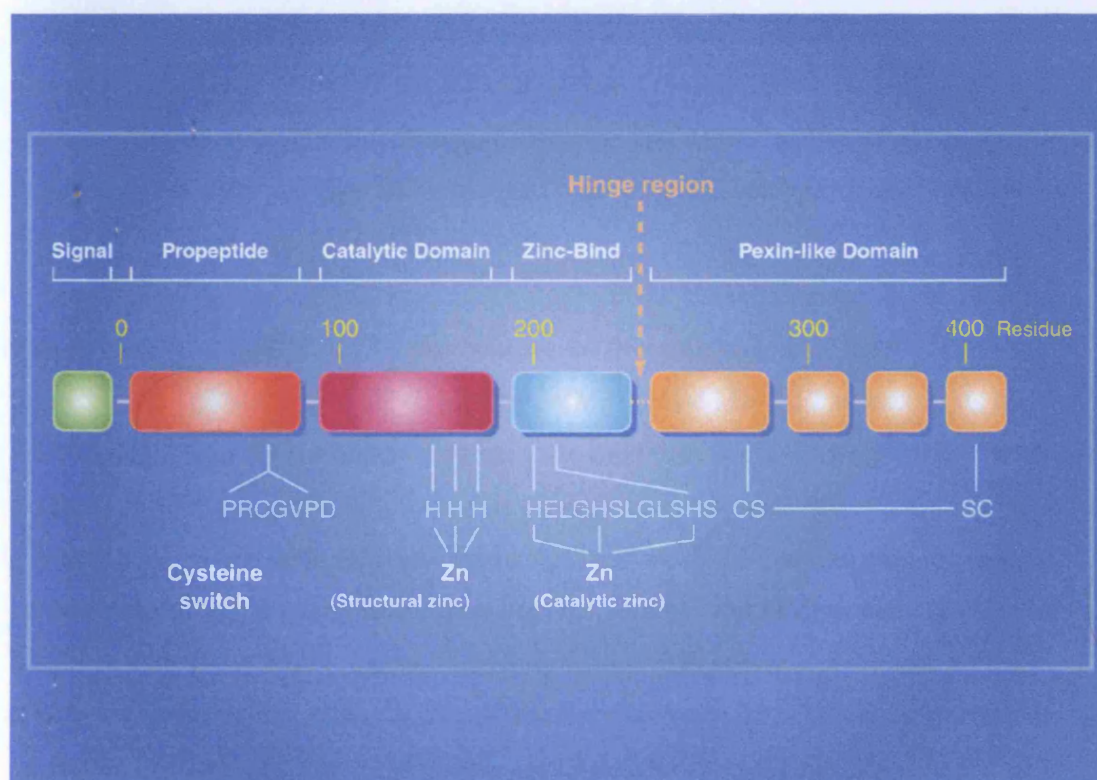


Figure 3.1 : The five domain structure of the MMP protein.

The hinge region is a small proline rich domain thought to allow folding of the c-terminal domain on to the catalytic domain. MMP-9 has a 54 amino acid insert in this region with homologies to the $\alpha 2$ chain of type V collagen (Wilhelm et al., 1989). The final domain

consists of 4 repeats with weak homology to hemopexin and vitronectin (Jenne and Stanley, 1987), the extremes of which are connected by a disulphide bridge. The function of this domain is unclear but is probably unimportant since both MMP-7 and MMP-12 function perfectly well without it.

MMP Genes

MMP genes also show a highly conserved modular structure. All collagenase and stromelysin genes are located in a gene cluster on the long arm of chromosome 11, with the exception of MMP-11 which has been mapped to chromosome 22 (Smith, 1993; Levy et al., 1992; Ye et al., 1998). The MMP-2 and MMP-9 genes reside respectively on chromosome 16 and 20 whereas the MT-MMP-1 gene is situated on chromosome 14 (Huhtala et al., 1990; Mignon et al., 1995). Comparison of the MMP genes has revealed a high degree of sequence homology, with each of the collagenase and stromelysin genes containing 10 exons and 9 introns spanning 8 to 12 kbp of DNA. The exception is MMP-7, the smallest MMP, which lacks exons 7-10 that encode the hemopexin-like domain, as well as all or most of exon 6 which encodes the hinge region (Birkedal-Hansen et al., 1993). Conversely, the genes for MMP-2 and MMP-9 are 26-27 kbp larger due to 3 additional exons which encode for the extended catalytic domain that specifies their gelatinolytic activity (Huhtala et al., 1990).

There are also considerable similarities between the promoter regions of the MMP genes, with those for MMP-1, MMP-3, MMP-7, MMP-9, MMP-10 and MMP-12 all containing one or more copies of the activator protein-1 binding site and the polyomavirus enhancer A-binding protein-3 site (Ye et al., 1998). These are very important in the regulation of gene expression, and their position relative to the transcription start site which differs from gene to gene, may account for the differences in observed expression patterns. The MMP-2 promoter lacks a TATA box and the activator protein-1 and the polyomavirus enhancer A-binding protein-3 sites, but contains a GC box for binding of transcription factor SP1. This unique promoter profile may well explain why the enzyme is constitutively expressed whereas the other MMPs are to a large extent inducible (Huhtala et al., 1990).

Site of MMP Production

The site of MMP production has been studied intensively by both immunocytochemistry and in situ hybridisation. MMPs are expressed in normal tissues with MMP-2 being the most

commonly expressed enzyme in normal adult tissues. However, the expression is limited to stromal cells (Matrisian, 1993). MMP-9 is actively expressed by haematopoietic cells (Hibbs et al., 1987) whilst MMP-7 is found in the glandular epithelial cells of the gastrointestinal tract and endometrium (Rodgers et al., 1993). It is also found in neoplastic cells (Newell et al., 1994), along with MT-MMPs on the tumour cell membrane (Sato et al., 1994). Most work on tumour tissue has shown that both the gelatinases and stromelysins are predominantly expressed by the stromal cells rather than tumour cells (Gallegos et al., 1995).

3.3 Regulation of Activity

Introduction

The activity of MMPs is tightly regulated at both an intra- and extra-cellular level (Figure 3.2). Intracellular regulation of gene expression occurs both by control of mRNA transcription and the half life of mRNA. MMPs are secreted in a latent form which require activation providing the first level of extracellular control of proteolysis. Furthermore, inhibition by TIMPs prevents activation and also directly inhibits the activity of enzymes.

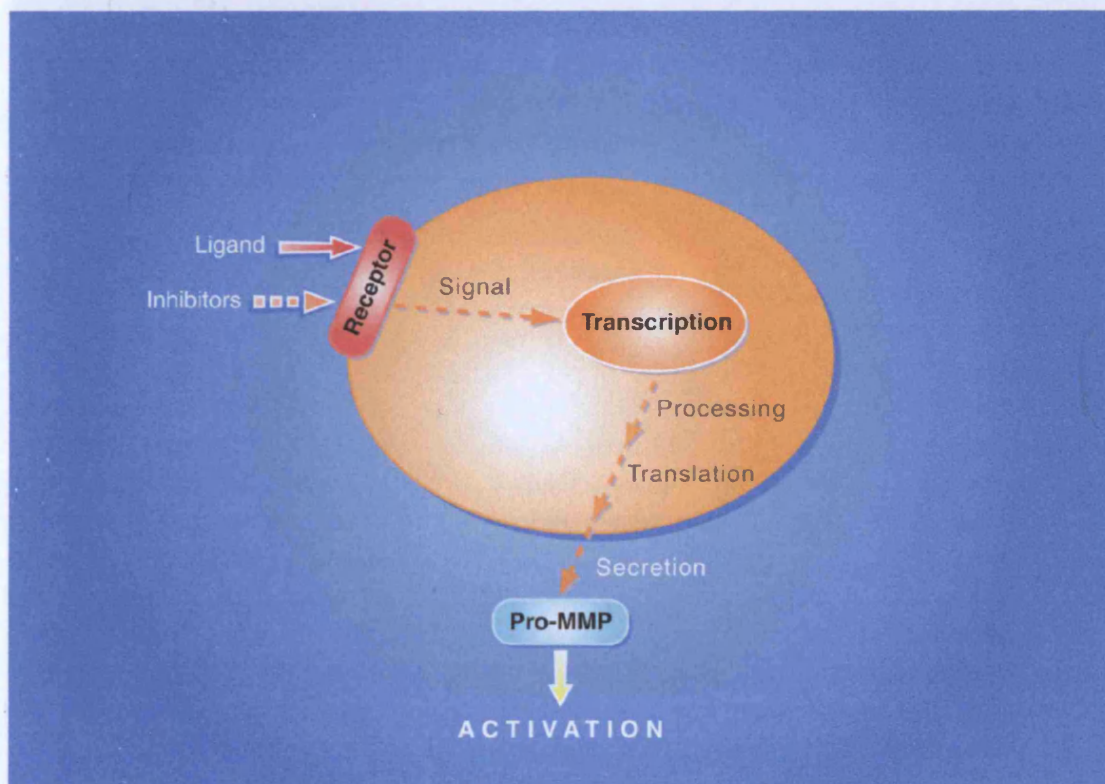


Figure 3.2: The control of MMP activity.

Control of Gene Expression

Many growth factors, cytokines, hormones and tumour promoters regulate MMP expression at the transcriptional level and a number of nuclear binding sites have been identified in the gene promoters as discussed previously. IL-1, PDGF and TNF- α have all been shown to have a stimulatory effect, whilst others such as heparin, TGF- β and corticosteroids have an inhibitory effect (Figure 3.3). However, despite different cell types possessing the same promoters, considerable variation exists in the response to cytokine stimulation. For example, in vitro studies have shown that TGF- β induces MMP-9 production in keratinocytes, has little effect upon smooth muscle cells and reduces production in fibroblasts (Kerr et al., 1988; Salo et al., 1991; Galis et al., 1994a). Clearly the precise mechanisms of action warrant further investigation.

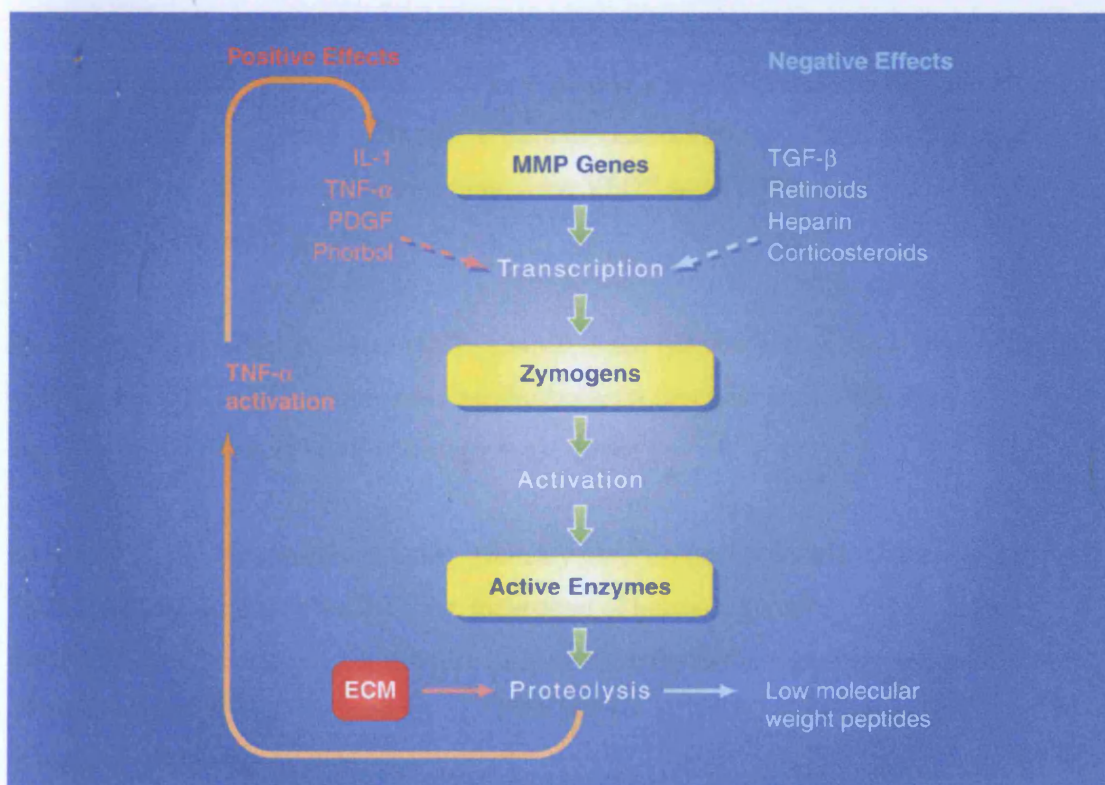


Figure 3.3: The control of MMP activity is complex, forming a positive feedback loop through the activation of TNF- α .

Specifically in human vascular smooth muscle cells, Galis and co-workers found that MMP-2 alone was expressed constitutively, but IL-1 and TNF- α induced the production of MMP-1, MMP-3 and MMP-9 (Galis et al., 1994a). They also found that both factors increased the activation of MMP-2 without affecting expression of the enzyme, nor altering the level or expression of TIMPs. Whilst this study failed to show any effect of TNF- β upon MMP expression, previous work has suggested a stimulatory role in cultured rabbit aortic smcs, and in smcs derived from human aneurysmal aorta with increased expression of MMP-1, MMP-3 and MMP-9 (Keen et al., 1994; Evans et al., 1991). Again there was no change in the transcription of TIMPs, suggesting the creation of an imbalance between proteases and inhibitors.

Vascular endothelial growth factor (VEGF) is a critical regulator of angiogenesis that stimulates migration, proliferation and proteolytic activity of endothelial cells. More recently it has been found to up-regulate the expression of MMPs in vascular smooth muscle cells (Wang and Keiser, 1998). In particular, VEGF stimulates the expression of MMP-1 and MMP-9 with a smaller increase in MMP-3, observed at both protein and mRNA levels. VEGF receptors are also found on monocytes and are known to mediate chemotaxis and cellular production of tissue factor, though a direct relationship between VEGF and MMP production in this cell type is yet to be established (Clauss et al., 1996).

Heparin has been shown to reduce MMP-1, MMP-3 and MMP-9 expression in baboon aortic smooth muscle cell explants stimulated by phorbol ester, but with little effect upon the expression of TIMP-1 or TIMP-2 (Kenagy et al., 1994).

Oxidised LDL, as previously discussed, probably plays an important role in atherogenesis by a variety of possible cellular mechanisms. Incubation of human macrophages with various concentrations of native ox-LDL produces a dose-response increase in expression of MMP-9 whilst down-regulating the expression of TIMP-1 (Xu et al., 1999). This is mirrored by an increase in gelatinolytic activity.

It is tempting to postulate that changes in the balance of certain cytokines and growth factors may alter the balance between MMPs and their inhibitors thus the activity of MMPs.

However, alterations in the mRNA and protein levels do not necessarily lead to a change in overall activity and further work is required to establish the role in vivo of these individual factors.

Extracellular Activation of the Pro-Enzyme

The second level of control of the MMPs is the extracellular activation of the secreted latent pro-enzymes. This is likely to be a crucial step in terms of their biological behaviour in disease states, since activation may tip the balance in favour of matrix degradation.

Activation occurs by cleavage of the pro-peptide, leading to a conformational change in the enzyme structure involving the cysteine switch mechanism. Essentially, in the latent form the cysteine residue in the highly conserved sequence PRCGV/NPD of the pro-domain interacts with zinc in the active site. Cleavage of the pro-peptide disrupts this relationship leading to activation of the enzyme(Springman et al., 1990).

The major physiological activator of the MMPs is plasmin, and the roles of urokinase plasminogen activator (uPA), which converts plasminogen to plasmin, and its receptor (uPA-r), have been studied in relation to tumour invasion and cell surface proteolysis(Liotta et al., 1991). The binding and activation of uPA on the cell surface receptor is thought to localise proteolytic activity to the leading edge of the cell(Kirchheimer and Remold, 1989). Both uPA and uPA-r are expressed by a variety of cells including those intimately involved in processes of tissue remodelling, in particular monocytes and macrophages. Furthermore, inhibition of uPA in vitro prevents matrix degradation(Estreicher et al., 1989).

The subsequent stages of activation form a similar positive feedback loop to the clotting cascade (Figure 3.4) whereby plasmin activates MMP-1, MMP-3 and MMP-9 directly, then active MMP-3 further activates MMP-1, active MMP-1 cleaves pro-MMP-9 and so on(Dollery et al., 1995). The action of MMP-3 upon MMP-1 leads to a 5-8 fold increase in proteolytic activity. Plasminogen activator inhibitor (PAI) has an inhibitory effect upon uPA, thus down-regulating the cascade leading to activation, and the balance between the two may be important in determining the overall level of proteolytic activity(Mauviel, 1993).

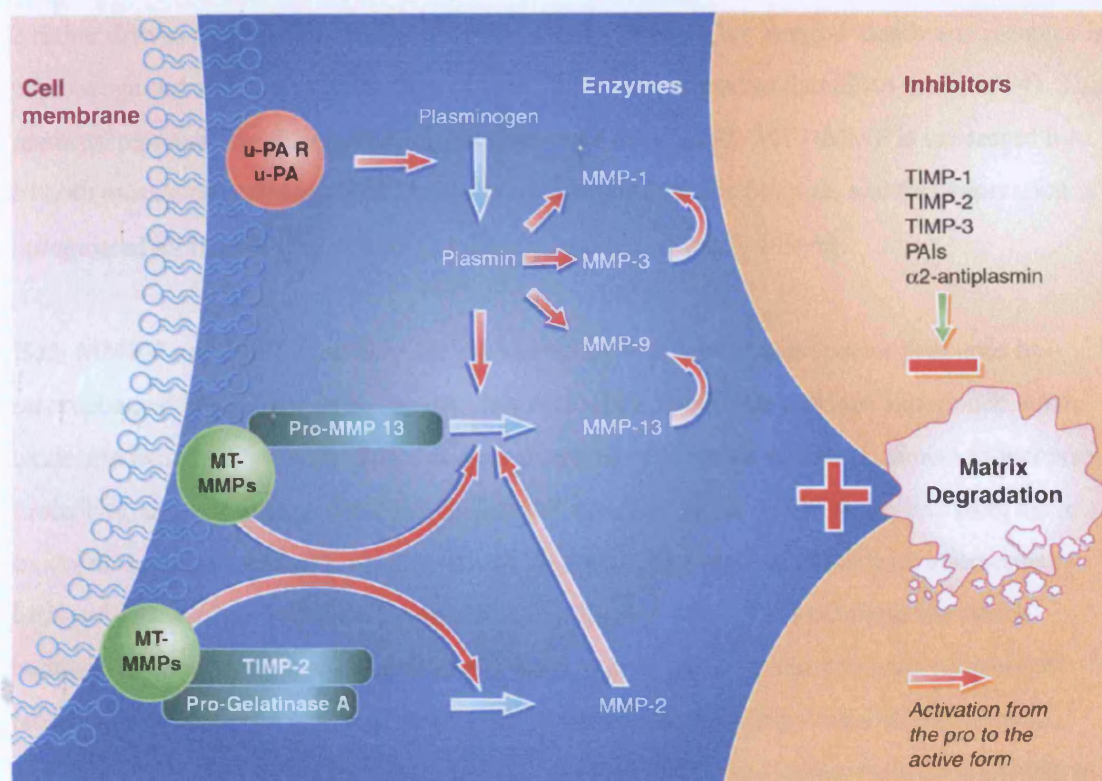


Figure 3.4: Interactions of various MMPs and other factors form a positive feedback cascade mechanism.

Other tissue factors including trypsin, chymotrypsin and plasma kallikrein can all activate MMP-s 1,3,8,9 and 10, though are thought to have a less significant role compared to plasmin (Suzuki et al., 1990a). Recently 'secretory leukocyte protease inhibitor', a serine protease inhibitor which has activity against trypsin, chymotrypsin, cathepsin G and neutrophil elastase, has been shown to down-regulate the production of MMP-1 and MMP-9 by human macrophages (Zhang et al., 1997). The mast cell derived proteases tryptase and chymase, released by in vitro degranulation of mast cells in human carotid endarterectomy specimens, also seem to cause an increase in overall MMP activity and matrix degradation (Johnson et al., 1998).

MMP-2 has proven resistant to activation in vitro by extracellular proteases, but cell membrane fractions of tumour cells were found to activate pro-MMP-2 by a mechanism sensitive to TIMPs (Ward et al., 1991). MMP-2 binds to a cell surface receptor when complexed at its hemopexin/vibronectin domain to the TIMP-2 c-domain. Activation also requires the presence of the COOH domain of either MMP-1 or MMP-3 as a target for TIMP-

2 rather than to take part in catalysis(Strongin et al., 1993). This integral membrane receptor is now recognised as MT-MMP of which 4 types have been cloned to date(Sato et al., 1994). This seems to represent the major physiological activator of MMP-2. MT1-MMP is expressed by smooth muscle cells and macrophages in human atherosclerotic plaques, and this expression is upregulated by IL1- α , TNF- α and ox-LDLs(Rajavashisth et al., 1999b).

Both MMP-2 and MMP-9 activity are modulated by reactive oxygen species produced by macrophage-derived foam cells. In vitro, isolated rabbit foam cells produce superoxide, nitric oxide and hydrogen peroxide. Superoxide and hydrogen peroxide have been shown to increase proteolytic activity, though the latter only at low concentrations, with little effect from nitric oxide(Rajagopalan et al., 1996). It is thought that they may react with the thiol interactions between cysteine residues in the pro-domain and the zinc atom of the catalytic site which maintain latency. Since it is recognised that there is an increase in the steady state levels of reactive oxygen species in a number of disease states including atherosclerosis(Ohara et al., 1993), it has been hypothesised that this may lead directly to increased activation of MMPs in plaques.

Tissue Inhibitors of MMPs

-Structure of TIMPs

The third regulatory mechanism involves the binding of the active MMP to TIMPs, and less specifically by α_2 -macroglobulin. The family of TIMPs include at least 4 members, TIMP-1 a glycoprotein weighing 28.5kDa, TIMPs 2-4 unglycosylated with a weight range 21-24kDa(Murphy and Docherty, 1992; Liu et al., 1997). Although structurally similar the TIMPs only share 37-42% amino acid identity, though are to a large extent interchangeable in their ability to inhibit MMPs. They are distinguished in their interactions with MMP-2 and MMP-9.

The TIMPs have an N and a C terminal domain, of which the former represents the inhibitory domain(Coulombe et al., 1988). Structural integrity is provided by six intra-molecular disulphide bridges which render the molecule highly stable(Williamson et al., 1990).

-Action of the TIMPs

The TIMPs inhibit MMPs by binding to the active form of the enzyme in a 1:1 molar ratio to form tight non-covalent complexes (Murphy and Willenbrock, 1995). This reaction in vivo is irreversible (Dollery et al., 1995). The TIMPs are themselves inhibited by trypsin, chymotrypsin and elastase but not by plasmin (Okada et al., 1988).

A more complex interaction exists between MMP-2 and TIMP-2, in which the C-domain of the inhibitor forms a complex with the haemopexin domain of the pro-enzyme, distinct from the inhibitory binding site; a similar relationship occurs between TIMP-1 and MMP-9 (Overall, 1994; Murphy and Willenbrock, 1995). The formation of these complexes prevents the activation of the bound latent enzyme whilst allowing the free N-terminal capable of inhibiting active unbound MMP. The interaction between MMP-2 and TIMP-2 is thought to be important in the activation of MMP-2 by MT1-MMP as described previously. The MMP-9/TIMP-1 complex becomes destabilised in the presence of excess MMP-3, the TIMP then binding MMP-3 and freeing the MMP-9 to be activated by further MMP-3, initiating the cascade process (Ogata et al., 1995). The net level of proteolytic activity is dependent upon the relative concentrations of the active enzymes and their inhibitors.

TIMP-1 is synthesised by most connective tissue cells and macrophages, the expression largely responsive to cytokines including IL-1, TGF- β and TNF- α (Overall, 1994). TIMP-2 expression, like MMP-2, is largely constitutive and follows a similar pattern to the enzyme with which it interacts (Stetler-Stevenson et al., 1993). The expression response of TIMP-2 to certain stimuli is in direct contrast to that for TIMP-1, suggesting differences in the regulatory elements of the gene promoters (Dollery et al., 1995).

TIMP-3, and particularly TIMP-4 are less well characterised. TIMP-3 is produced by smooth muscle cells and macrophages and, once secreted, binds to and forms an insoluble complex with constituents of the ECM (Pavloff et al., 1992). The expression is up-regulated by PDGF and TGF- β (Fabunmi et al., 1998). TIMP-4 has a similar spectrum of inhibitory activity but with a particular interaction with MMP-2 (Bigg et al., 1997; Wang et al., 1999).

3.4 Summary

The balance between expression of MMPs and TIMPs controls the overall composition of the extracellular matrix. In disease states, over-expression of MMPs in relation to TIMPs may lead to a situation of net matrix degradation, whilst the converse may also be true leading to overall matrix accumulation, or at least prevention of degradation. However, in healthy tissues they seem to form a linked cascade with a parallel, tightly regulated system.

MMP	Enzyme	Principal known substrates
Collagenases		
MMP-1	Interstitial collagenase	Collagens I,II,III,VII,VIII,X, gelatin, aggrecan, MMPs 2 and 9
MMP-8		Collagens I,II,III,V,VII,VIII,X, gelatin, aggrecan
MMP-13	Neutrophil collagenase	Collagens I,II,III,IV, gelatin, aggrecan, PAI2
MMP-18	Collagenase-3 Xenopus collagenase	
Gelatinases		
MMP-2	Gelatinase A (72 kDa gelatinase)	Gelatin, collagen types I,IV,V,VII,X,XI,XIV, elastin, fibronectin, aggrecan.
MMP-9	Gelatinase B (92 kDa gelatinase)	Gelatin, collagen types IV,V,VII,X, elastin.
Stromelysins		
MMP-3	Stromelysin-1	Collagens III,IV,IX,X, gelatin, aggrecan, MMPs 1,7,8,9 and 13.
MMP-10	Stromelysin-2	Collagens III,IV,V, gelatin, casein, MMPs 1 and 8
MMP-11	Stromelysin-3	
MMP-7	Matrilysin (PUMP-1)	Collagens IV,X, gelatin, fibronectin
MMP-12	Macrophage metalloelastase	Collagen IV, gelatin, elastin, fibronectin
Membrane type MMP's		
MMP-14	MT-MMP-1	Collagens I,II,III, gelatin, MMPs 2 and 13
MMP-15	MT-MMP-2	MMP-2, gelatin
MMP-16	MT-MMP-3	MMP-2
MMP-17	MT-MMP-4	

Table 3.1: *The major MMP subtypes and their substrates*

CHAPTER FOUR

THE ROLE OF MMPs IN VASCULAR DISEASE

4.1 Introduction

4.2 MMPs in Aneurysmal Disease

Histological Changes in Aneurysms

Gelatinases in Aneurysms

Systemic Changes In Gelatinase Levels

Other MMPs in Aneurysmal Disease

TIMPs in Aneurysms

Summary

4.3 MMPs in Intimal Hyperplasia

The Histology of Intimal Hyperplasia

The Role of MMPs

TIMPs and Intimal Hyperplasia

Summary

4.4 MMPs in Atherosclerosis

Early Plaque Development

Plaque Disruption

The Role of MT1-MMP

Evidence of Increased Proteolysis

Genetic Factors

Summary

4.1 Introduction

The recognition that MMPs and their balance with inhibitors control the regulation of the extracellular matrix has led to considerable interest in their pathological effect in vascular disease states, where tissue remodelling plays such an important role. There is a growing body of evidence supporting a pivotal role for MMPs in 3 broad areas of vascular disease, namely aneurysm formation, intimal hyperplasia and atherosclerosis. This has led to the study of MMP inhibition in models of vascular disease with promising results.

4.2 MMPs in Aneurysmal Disease

Histological Changes in Aneurysms

Aneurysms are defined as a 50% increase in the diameter of an artery compared to the expected normal value (Johnson et al., 1991). The commonest site for aneurysms is the infrarenal aorta, with a prevalence in England of up to 5% (Collin et al., 1988) (Figure 4.1). Despite surgical intervention, abdominal aortic aneurysms account for 1.5% of the total mortality in males over 55 years old (Hak et al., 1996).

Aneurysmal disease is characterised by extensive medial degeneration with loss of elastin from both media and adventitia (Baxter et al., 1992), apoptosis and reduced numbers of matrix synthesising smooth muscle cells (He and Roach, 1994). There is also an inflammatory infiltrate consisting of T-cells, B-cells and macrophages (Koch et al., 1990). Inflammation and excessive breakdown of the extra cellular matrix have been identified as the cause of destruction of the arterial wall (Shah, 1997).

The most important structural elements of the aortic wall are collagen types I and III and elastin. Collagen fibres are arranged in such a way that, while normally extremely resistant to stretch, the vessel is elastic with the main load placed upon the elastin fibres (Clark and Glagov, 1985). It is thought to be elastin degradation that initiates the expansion of the vessel, with loss of fibrillar collagen predisposing the aneurysm to rupture (Dobrin and Mrkvicka, 1994). The loss of elastin is a striking histological feature of the aneurysm wall, representing only 8% of the dry weight compared to 35% of non-aneurysmal aorta (Campa et al., 1987). Elastin is not

synthesised in the aortic wall, having a half-life of up to 70 years, so loss of elastin is recognised as a manifestation of excessive elastolysis(Powell et al., 1992). Infusion of elastase in animal and in-vitro human models leads to aneurysm formation, intense inflammatory infiltration, loss of medial elastin and the overproduction of proteolytic enzymes(Wills et al., 1996b; Anidjar et al., 1990; Anidjar et al., 1992; Halpern et al., 1994). Furthermore, diseases such as Marfans and Ehlers Danlos type IV disease where mutations in genes encoding type III collagen and fibrillin-I, an elastic fibre component, predispose individuals to aneurysm formation(McKusick, 1991). The majority of aneurysms however develop in individuals as an acquired degenerative disorder without identified genetic defects(Anidjar and Kieffer, 1992).

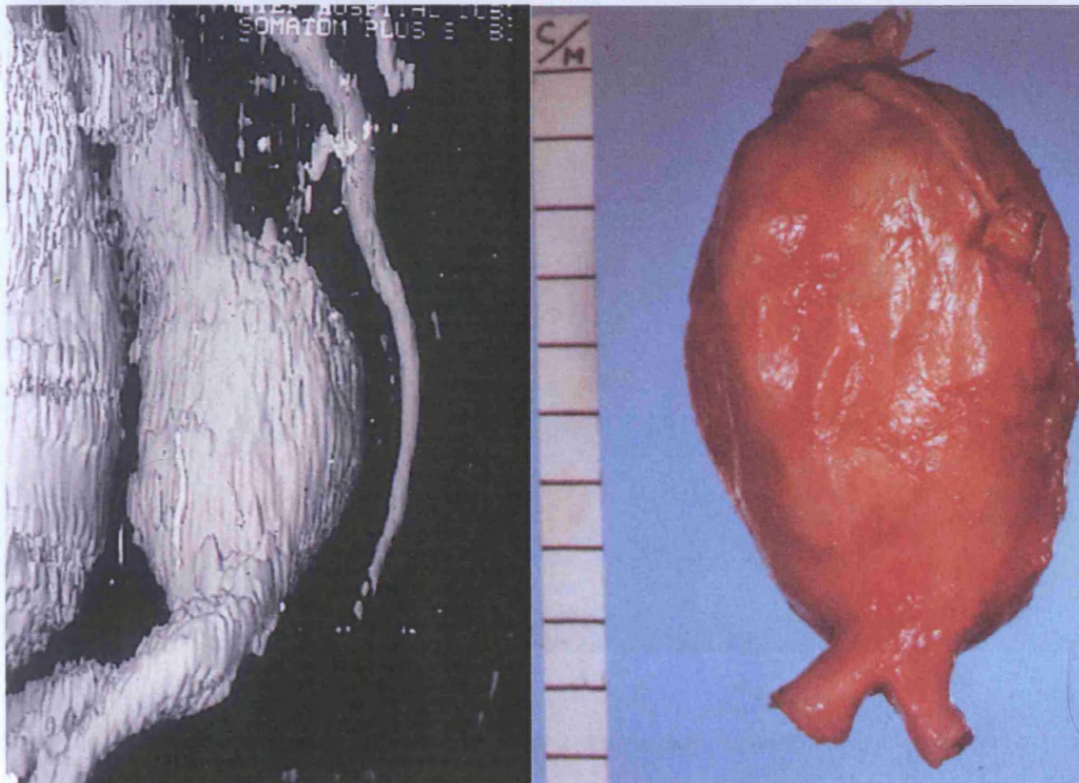


Figure 4.1: *Abdominal aortic aneurysms, such as the ones shown in this CT angiogram and post-mortem specimen, dilate progressively and may rupture.*

Interestingly the collagen content of aneurysms increases with size(Minion et al., 1994; Halloran and Baxter, 1995). This is thought to be a compensatory mechanism to increased wall stress(White et al., 1993) since this is known to stimulate matrix synthesis by smooth muscle cells in vitro(Leung et al., 1976). Messenger RNA levels for the individual matrix constituents

have been investigated in the aneurysm wall, and procollagen expression is significantly increased compared to non-aneurysmal tissue (McGee et al., 1991). It therefore seems likely that a combination of increased collagen synthesis with elastin degradation combine to alter the matrix structure of the vessel wall in aneurysms (Wills et al., 1996a), with eventual failure of both contributing to rupture (Figure 4.2).

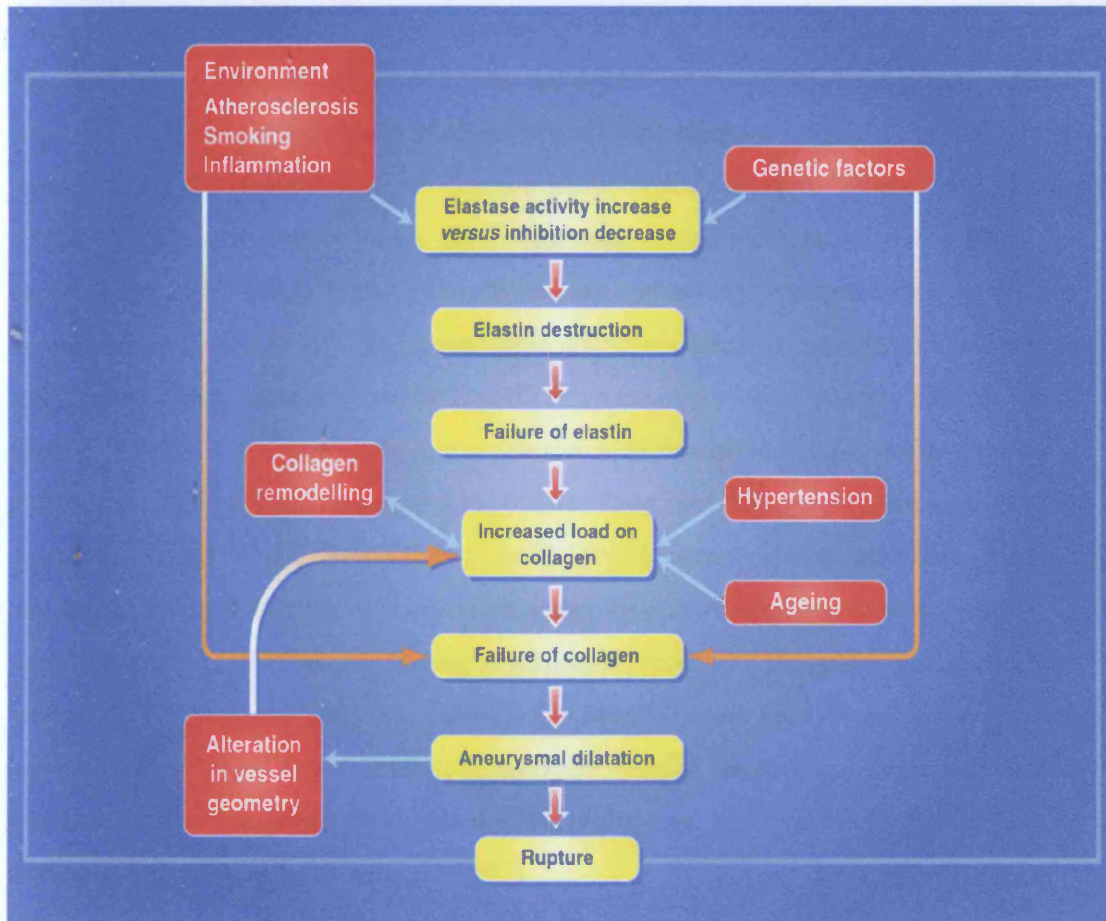


Figure 4.2: Proposed chain of events leading to aneurysmal dilatation and eventually rupture.

Gelatinases in Aneurysms

Interest in the role of proteolytic enzymes in aneurysm development has grown considerably since the first reports by Busuttil and Campa in the 1980s (Busuttil et al., 1980; Campa et al., 1987). They described an increase in elastase and collagenase activity within aneurysmal tissue and identified a 90-kDa elastase of the MMP family, now recognised as MMP-9. Vine and

Powell reported an increase in gelatinolytic activity, associated with a spectrum of enzymes of molecular weights ranging from 55-92 kDa(Vine and Powell, 1991). They also detected stromelysin and collagenase activity, though the levels were relatively small. Brophy published similar results regarding gelatinolytic activity in aneurysmal tissue in the same year(Brophy et al., 1991).

Tissue extracts from aneurysms exhibiting high levels of gelatinase activity are capable of degrading elastin in tissue sections of human aorta(Reilly et al., 1992). Subsequent studies have confirmed that the high levels of elastolytic activity represent a combination of increased levels of MMP-2(McMillan et al., 1995b) and MMP-9(Newman et al., 1994; Thompson et al., 1995), both of which degrade elastin, the former being the more potent enzyme in this regard(Senior et al., 1991). In particular, Thompson and co-workers demonstrated a 10 fold increase in the level of MMP-9 in human aortic aneurysm tissue compared to normal controls, and localised the expression to macrophages(Thompson et al., 1995). Further work by the same group studied the ratio of enzyme expression to their inhibitors using competitive polymerase chain reaction(Tamarina et al., 1997). They identified a significant increase in the expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 in aneurysms compared to normal aorta, but also a significant increase in the ratio between enzyme and inhibitor in aneurysms, suggesting a potential imbalance leading to increased proteolysis. The predominant enzyme, confirming the earlier work described above, was MMP-9, with mRNA levels 20 times and 2 times higher than MMP-1 and MMP-2 respectively. Similar results were obtained by Knox who demonstrated a significant increase in the ratio between MMP-2 and TIMP-2 in aneurysm compared to normal aorta(Knox et al., 1997).

This hypothesis has been studied further in vitro. When monocytes were cultured with aortic explants, they infiltrated the tissue, produced MMP-9 and initiated proteolytic destruction of the elastin component(Katsuda et al., 1994). This phenomenon was prevented by the addition of MMP-9 inhibitors. Others have demonstrated high levels of MMP-9 in aneurysmal aorta, predominantly localising to macrophages(McMillan et al., 1995a; Freestone et al., 1995), and in contradistinction to normal tissue a significant proportion of MMP-2 and MMP-9 are found in the active forms (Sakalihasan et al., 1996). However, smooth muscle cells may also play a role in enzyme production. Patel et al isolated aortic smooth muscle cells from patients with aneurysmal disease and examined the production of MMP-2 and -9 by Western blotting and gelatin zymography(Patel et al., 1996). Medial smooth muscle cells from aneurysmal tissue produced significantly higher levels of both MMP-2 and MMP-9 than control cells.

More recently, Davis and colleagues have provided further information regarding the expression of MMP-2 and -9 within aneurysms (Davis et al., 1998). They employed a combination of gelatin zymography and competitive RT-PCR to study the production and expression within human aortic tissue harvested from patients undergoing bypass surgery for aneurysms or occlusive disease and compared them to non-diseased tissue from organ donors. The level of MMP-9 was significantly higher in both aneurysmal and occlusive disease compared to controls, but with little difference between the aneurysm and occlusive groups themselves. The predominant source of the enzyme was macrophages. In contrast, the level of MMP-2 was significantly higher in aneurysms compared to both normal aorta and occlusive disease, with mesenchymal cells forming the primary source of enzyme. Interestingly, MMP-2 production was predominantly by mesenchymal cells surrounded by macrophages, suggesting a paracrine effect upon MMP-2 expression. Furthermore, a large proportion of the MMP-2 was in the active form, tightly bound to the tissue matrix.

As discussed previously, MT1-MMP is involved in the processing of the MMP-2 pro-enzyme. MT1-MMP is expressed in the aneurysm wall by both macrophages and smooth muscle cells in a similar pattern to MMP-2. This indicates a broader distribution of expression than in occlusive disease and potentially a different role in the control of matrix degradation between the 2 degenerative aortic diseases (Thompson and Parks, 1996). Though the interaction between smooth muscle cells and inflammatory cells and their relative importance in aneurysmal disease requires clarification, it seems likely that both are intricately involved the proteolytic cascade leading to tissue degradation (Shah, 1997).

Systemic Changes In Gelatinase Levels

While the majority of work regarding MMP-2 and MMP-9 has focused upon the abdominal aorta, increased proteolysis may be a systemic phenomenon. Recent studies have shown an association between serum MMP-2 levels and the occurrence of cerebral aneurysms (Todor et al., 1998). Interestingly, an association has also been made between the development of intracranial aneurysms and a functional polymorphism in the MMP-9 promoter region, possibly leading to variability in expression at the level of transcription (Peters et al., 1999). The plasma level of MMP-9 is increased in patients with abdominal aortic aneurysms (McMillan et al., 1999). In a study by McMillan and colleagues, plasma MMP-9

levels in patients with occlusive disease and controls were similar, but significantly higher in those with aortic aneurysms. Furthermore, the level in those patients with multiple aneurysms was significantly higher than in those with isolated aortic aneurysms. It is unclear whether the aneurysms represented the source of the increased circulating levels and the relationship between these observations and the pathogenesis of aneurysms requires clarification.

Other MMPs in Aneurysmal Disease

Whilst Busuttil in his early report demonstrated increased collagenolytic activity in aneurysms (Busuttil et al., 1980), a further 10 years passed before Vine and Powell demonstrated the presence of MMP-1 in aneurysms using specific antisera (Vine and Powell, 1991). Irizarry et al reported increased levels of MMP-1 in extracts of aortic aneurysm compared to normal aorta using immunoblotting (Irizarry et al., 1993), and immunofluorescent studies localised the enzyme to mesenchymal cells in the adventitia, including endothelial cells. They proposed that collagen failure was a sentinel event in aneurysm formation (Tilson et al., 1990). Much of the collagen is situated within the adventitia (Davidson et al., 1985), and the presence of increased MMP-1 in the adventitia could be important in aneurysm progression.

Whilst the gelatinases seem to be the most important MMPs with regard to elastolysis in aneurysm pathogenesis, there is increasing evidence linking other members of the family to the disease. MMP-8, identified within atherosclerotic disease and produced by macrophages, has also been identified in aneurysms (Thompson and Parks, 1996). MMP-12, also initially identified in occlusive disease, was expressed in complex aneurysmal aortic lesions in apo-E deficient mice (Carmeliet et al., 1997). More importantly, recent work identified MMP-12 in human aneurysmal tissue, expressed by infiltrating macrophages and localised to fragmented aortic elastin products (Curci et al., 1998b). This pattern is distinct from other elastolytic enzymes and suggests a particularly important role in matrix degeneration. The level of MMP-12 was 7 times higher than normal aorta.

MMP-13, a collagenase with limited tissue distribution and a tightly regulated pattern of expression, has been identified in human aortic aneurysms (Mao et al., 1999). Using RT-PCR and Southern blots, MMP-13 was detected in both aneurysmal tissue and occlusive disease but not normal control tissue, with a higher level of expression in aneurysmal tissue compared to

atherosclerotic aorta. MMP-13 was localised to smooth muscle cells which, when isolated and cultured, continued to express mRNA for MMP-13 in vitro.

MMP-3 has also been identified within aneurysms as well as cultured smooth muscle cells from aneurysmal tissue(Newman et al., 1994; Keen et al., 1994). Whilst having the ability to degrade various constituents of the extracellular matrix, the role of the enzyme in aneurysm pathogenesis, where elastin and collagen degradation play such a key part, is unclear. Its relevance in the proteolytic cascade may lie in the activation of MMP-1 (Suzuki et al., 1990b).

TIMPs in Aneurysmal Disease

Contrary to atherosclerotic disease, there is little to suggest an imbalance between the levels of proteolytic enzymes and their inhibitors in aneurysms. Although early data suggested a decrease in the level of TIMPs in aneurysms(Brophy et al., 1991), this was not confirmed in subsequent studies(Tilson et al., 1993). Indeed many studies have shown the opposite to be true, with increased levels of TIMP-1 being demonstrated(Thompson et al., 1995; McMillan et al., 1995a). Despite this, local over-expression of TIMP-1 by transfection of TIMP-1 DNA to smooth muscle cells in a rat model of aneurysmal disease prevented rupture(Allaire et al., 1998) and a variant in the TIMP-2 coding sequence has been associated with the identification of aneurysms in humans(Wang et al., 1999).

Summary

In summary, numerous studies have provided evidence that MMPs, particularly those with elastolytic and collagenolytic activity, are important molecular participants in aneurysm pathogenesis. However, their precise roles and the potential for pharmacological intervention will require further clarification. The reasons why certain individuals should be prone to matrix degeneration and aneurysm formation remain elusive, and the pathogenesis is certainly multifactorial (Figure 4.2)(MacSweeney et al., 1994). There is a genetic component, since first degree relatives of individuals with aneurysms are at an increased risk of developing a similar lesion compared to the general public, independent of other known cardiovascular risk factors(Tilson, 1992; Baird et al., 1995). Smoking is the single largest environmental risk factor, others including hyperlipidaemia and hypertension, but their precise role remains

unclear. A better understanding of the interaction between inflammatory cells and mesenchymal cells within the arterial wall should allow identification of specific genetic risk factors which predispose to aneurysm development.

4.3 MMPs in Intimal Hyperplasia

The Pathophysiology of Intimal Hyperplasia

Intimal hyperplasia occurs in damaged arteries, arterialised vein and prosthetic bypass grafts. This process is characterised by the migration and proliferation of smooth muscle cells with subsequent accumulation of extracellular matrix, producing luminal narrowing (Figure 4.3). Intimal hyperplasia occurs between the endothelium and the internal elastic lamina in arteries or between the endothelium and medial smooth muscle cells in vein grafts, with distinct hyperacute, acute and chronic phases (Davies and Hagen, 1994).

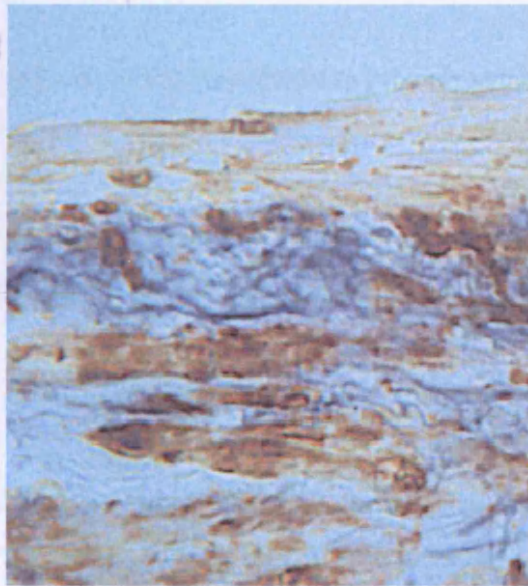


Figure 4.3: *Intimal hyperplasia, characterised by smooth muscle cell proliferation and extracellular matrix accumulation.*

Endothelial cell damage triggers a complex series of events leading to a phenotypic change in smooth muscle cells, from a contractile to secretory role (Dilley et al., 1988). These cells undergo a period of proliferation and migration from the media to the intima where they

undergo a second proliferative phase, followed by a quiescent stage of matrix synthesis. These changes are responsible for the majority of late failures following bypass grafting(Sayers et al., 1993), angioplasty(Mata et al., 1985) and endarterectomy(Johnson et al., 1990; Schwartz et al., 1987).

Much of our understanding of intimal hyperplasia has been gained from animal models of vascular injury(Clowes et al., 1983). Intimal injury leads to denudation of the endothelium and medial necrosis and recovery can take up to 12 weeks. Up to 25% of the innermost medial smooth muscle cells are lost following injury but cellular proliferation starts within 48 hours, returning to normal levels after approximately 4 weeks. Meanwhile, endothelial regeneration is initiated from the undamaged ends. Migration of smooth muscle cells from the media to the intima occurs 4 days following injury, and upon reaching the intima they undergo further proliferation. The excess proliferation probably represents an exaggeration of the normal healing response, largely regulated by growth factors such as basic fibroblast growth factor (bFGF) and PDGF which control smooth muscle cell replication and migration respectively(Ferns et al., 1991; Olson et al., 1992).

Human venous endothelium has been shown to promote neointimal formation through a paracrine action upon the vascular smooth muscle cell(Allen et al., 1994). Platelet aggregation and interactions between leukocytes and endothelial cells may also be involved, possibly through membrane glycoproteins called 'selectins'. P-selectin deficient mice fail to initiate an inflammatory response to vessel wall injury, nor develop a neointima(Kumar et al., 1997).

The breaching of the basement membrane by migrating smooth muscle cells to reach the intima appears to be the rate-limiting step in intimal hyperplasia. There is mounting evidence that MMPs control this stage of the process and are intricately involved in the pathogenesis of stenoses through their expression by smooth muscle cells.

The Role of MMPs

In vitro models have been used to demonstrate increased smooth muscle cell expression of collagenase and stromelysin genes following mechanical injury(James et al., 1993), and this is now supported by work in animal models. In studies of the rat carotid artery, there was constitutive expression of MMP-2 and a marked increase in activity between 4 and 14 days

after arterial injury(Bendeck et al., 1994). MMP-9 expression was also increased but this occurred early after injury, peaking at 3 days. Administration of an MMP inhibitor resulted in a 97% reduction in smooth muscle cell migration. The authors suggested that active MMP-9 might be controlling migration of smooth muscle cells from media to intima. In contrast, mature restenotic lesions with abundant extracellular matrix exhibit reduced MMP activity and increased levels of TIMP-1 compared to both normal artery wall and atherosclerotic lesions(Tyagi et al., 1995). The increased expression of extracellular matrix proteins occurred within 7 days of injury, and while the activity of MMPs declined to low levels, the expression of tropoelastin remained at a high level for more than 28 days(Matsuoka et al., 1998).

Studies in a pig model of arterial injury revealed a similar response, with MMP-9 induced to a peak at 3 days and MMP-2 at 7 days, though levels remained elevated until 21 days(Southgate et al., 1996). Expression was localised to medial smooth muscle cells, particularly around areas of injury-induced necrosis. In this model, the upregulation of MMP production paralleled the time course of smooth muscle cell migration and proliferation. Use of vein as carotid interposition grafts in the pig has shown a similar up-regulation of both MMP-2 and MMP-9 expression, again localised to the proliferative smooth muscle cells at the base of the developing neointima(Southgate et al., 1999).

There is evidence that haemodynamic factors such as low flow and shear stress are associated with the localisation and progression of intimal hyperplasia. Studies in rabbits showed that low flow upregulated the injury-induced MMP-2 expression, through either a cytokine mediated pathway, changes in platelet aggregation or a direct difference in smooth muscle cell stimulation in response to shear stress(Bassiouny et al., 1998). The authors suggested that haemodynamic factors might be more important than the injury itself.

Further in vitro studies have shown that the activation of MMP-2 was crucial for the migration of vascular smooth muscle cells through a reconstituted basement membrane similar in composition to that surrounding the cells in vivo(Pauly et al., 1994). This has led to interest in the role of MT1-MMP, and further work in the rat carotid artery injury model showed a 6 fold increase in MT1-MMP mRNA levels 3 days after injury. This coincided with an increase in activation of MMP-2 detected on gelatin zymography, while the total MMP-2 protein level reached a peak 5-7 days after injury, localised to the developing neointima. Similarly, MMP-9 overexpression by transfection of smooth muscle cells with a cDNA for rat MMP-9, increased cell migration in a collagen invasion assay and Boyden chamber in vitro(Mason et al., 1999).

Extending this work to the in vivo animal model similarly increased cell migration and altered arterial remodelling.

Interestingly, transfection of vascular smooth muscle cells in culture with endothelial nitric oxide synthase gene inhibited cell migration, mediated by altered expression of MMPs(Gurjar et al., 1999). A significant increase in the expression of TIMP-2 was observed, while the activity of both MMP-2 and MMP-9 was significantly reduced. The relevance of this to intimal hyperplasia in the human and the potential for therapeutic prevention is unclear.

In a primate model, positive immunostaining for MMP-1, -2, -3 and -9 appeared in the developing neointima throughout the first week following balloon injury(Aoyagi et al., 1998). Early on, staining for MMPs -1, -3 and -9 was localised to replicating cells, eventually confined to the luminal surface of the neointima. MMP-2 positive cells appeared in the basal layer of the neointima 2 weeks post-injury and throughout the subsequent 4 weeks came to occupy the entire neointima. These cells were negative for proliferation-associated antigens and became surrounded by matrix proteins. This suggested a co-ordinated MMP response to the initial injury, but a later stage dependent upon MMP-2 for facilitating further migration but not proliferation of cells within the neointima. Evidence suggests that primate smooth muscle cell migration in vitro is regulated by PDGF and bFGF, with PDGF induced migration dependent upon MMP-2 and bFGF actions dependent upon both MMP-2 and MMP-9(Kenagy et al., 1997).

Using human long saphenous vein in vitro, injury induced the expression of MMP-9, especially in the proliferative neointimal smooth muscle cells, and also increased the levels of both active and latent MMP-2(George et al., 1997). In this model, intact vein developed a significantly thicker neointima than denuded vein(Porter et al., 1999). Co-culture with an MMP inhibitor prevented neointimal formation in conjunction with a significant decrease in the levels of MMP-2 and MMP-9(Porter et al., 1998).

TIMPs and Intimal Hyperplasia

Little is known about the role of TIMPs in intimal hyperplasia, but models of intimal hyperplasia in which TIMP expression is increased have shown a reduction in neointimal formation. Expression of TIMP-1 by the use of an adenoviral vector to transfect rat smooth muscle cells reduced intimal hyperplasia in the rat arterial injury model(Dollery et al., 1999a).

Interestingly, longer term the TIMP-1 over-expression led to changes in post-translational processing of elastin, with excessive accumulation of cross-linked elastin(Forough et al., 1998). Similar results were observed in mice when the 'wild-type' mouse was compared to a TIMP-1 deficient, where increased neointima was detected following electrical insult to the femoral artery in tandem with increased levels of MMP-2 and MMP-9 activity(Lijnen and Collen, 1999). This has been studied in the human in vitro model of saphenous vein culture, where adenovirus mediated gene transfer of the human TIMP-1 gene inhibited smooth muscle cell migration and neointimal formation(George et al., 1998). There was no effect upon cell proliferation or viability, and a specific MMP inhibitory effect has been shown using in situ zymography.

TIMP-2 has been shown to have a dose-dependant effect in reducing cell proliferation and TIMP-3 promoted smooth muscle cell death by apoptosis(Baker et al., 1998). These effects were independent of their inhibitory actions on MMPs and were not reproduced by the addition of synthetic MMP inhibitors. Clearly this has implications for potential therapies based on TIMPs or gene therapy. In the human model, TIMP-1 and TIMP-2 secretion were increased in the early stages following injury with TIMP-2 remaining partially attached to extracellular matrix(Kranzhofer et al., 1999). TIMP-3 was detected before culture and expression was variable, detectable throughout periods in culture and remaining exclusively attached to the extracellular matrix. TIMP-4 expression was also increased following injury and reduced smooth muscle cell migration(Dollery et al., 1999b). The regulation of TIMP expression seems to be an important net determinant of MMP activity in neointima formation, and appears to restrict it to the pericellular environment.

Summary

It is clear that the smooth muscle cell can alter its surrounding matrix by the production and secretion of MMPs and their inhibitors, the balance of which regulates net matrix accumulation or destruction. Early after injury, migration into the intima seems to be the key step, initiating neointimal formation by proliferation and matrix protein synthesis. MMP inhibition seems to prevent this, but so far this is only established in animal models and in vitro culture of human vein. Other proteolytic factors may be involved including serine proteases, since their inhibition has also prevented intimal hyperplasia(Lucas et al., 1996). This may have major clinical

implications for therapy directed at preventing intimal hyperplasia and reducing restenosis and graft failure, with the MMP/TIMP family representing an attractive target.

4.4 MMPs in Atherosclerosis

Early Plaque Development

Because of their key role in matrix turnover, MMPs have a potential role in many aspects of vascular biology. In atherosclerosis they may be involved in the early stages of plaque development but in particular the weakening of plaque caps prior to fissuring or rupture.

Monocyte adherence to the vessel wall is one of the earliest events in atherogenesis. On reaching the subintimal space, complex cell-cell interactions occur with the release of cytokines and growth factors, as described in Chapter 1. Plaque formation occurs as the result of cellular migration and proliferation accompanied by an accumulation of ecm. Vascular smooth muscle cell migration is dependant upon the production of MMP-2, the limiting step being the degradation of basement membrane, composed of type IV collagen. In a study by Pauly, isolated rat vascular smcs cultured on a gel of reconstituted basement membrane proteins secreted MMP-2 which was activated to degrade the proteins(Pauly et al., 1994). Antiserum against MMP-2 prevented migration through the basement membrane. Similar results have been observed in a rabbit model though direct evidence of an association in the human is lacking(Southgate et al., 1992). However this appears to be the first limiting step in atherogenesis controlled, at least in part, by MMPs.

Plaque Disruption

There is also increasing evidence to suggest that MMPs are intricately involved in the destruction of connective tissue prior to surface disruption and thrombo-embolism. Early work by Vine and Powell demonstrated an increase in gelatinase activity in both occlusive and aneurysmal aortic disease compared to control(Vine and Powell, 1991). In occlusive aortas zymography suggested that this was predominantly MMP-9. They also demonstrated collagenase and stromelysin activity in similar tissue, though this was not quantified. Henney described the presence of MMP-3 mRNA in human coronary atheroma by in situ hybridisation,

localised to areas of intense macrophage infiltration in the shoulder of the plaque(Henney et al., 1991)

Subsequently Galis studied the expression of a variety of MMPs in human atherosclerotic plaques, and used in situ zymography to assess matrix-degrading activity(Galis et al., 1994b). In non-diseased arterial tissue, smooth muscle cells expressed MMP-2 as well as TIMPs -1 and -2. In contrast, atherosclerotic lesions showed immunoreactivity for MMPs -1, -2, -3 and -9 as well as the TIMPs, localising -1, -3 and -9 to macrophages, lymphocytes, smooth muscle cells and endothelial cells within the fibrous cap and shoulder of the plaque. In situ zymography demonstrated gelatinolytic and caseinolytic activity within the same areas suggesting a potential role for these enzymes in plaque matrix degradation. The levels and expression of the enzymes were not quantified in this study.

Further work on MMP-1 showed that expression in human carotid plaques retrieved following endarterectomy was predominantly by macrophages in the cap and shoulder regions, though subsets of smooth muscle cells and endothelial cells also express MMP-1(Nikkari et al., 1995). Furthermore, they demonstrated a strong correlation between the percentage of the lipid core staining positively for MMP-1 and the percentage of core occupied by haemorrhage, evidence of plaque instability. Whilst the numbers were small, this represented the first attempt to relate MMP production with features of instability. No attempt was made to quantify activity nor enzyme levels.

Brown and co-workers investigated the role of MMP-9 in acute coronary ischaemia(Brown et al., 1995). They performed immunostaining for MMP-9 upon atherectomy specimens retrieved from 12 patients with unstable angina and compared them to a similar cohort with stable angina. They reported positive staining in 85% of both groups. However, intracellular staining was seen in all of those with positive staining in the unstable angina group, compared to 3/10 of the stable groups. This provided evidence of active enzyme synthesis, since macrophages do not store MMP-9(Hibbs, 1992). The predominant cell types were identified as macrophages and smooth muscle cells. They concluded that active synthesis of the enzyme was more common in coronary plaques undergoing acute disruption, causing unstable angina. They recognised certain limitations of the study, particularly the lack of evidence of increased activity and the absence of data regarding TIMP levels. The study involved small numbers and failed to provide sufficient histological evidence to support their claim. Though not truly

quantitative, the study raised awareness of the potential role for MMP-9 in acute plaque disruption.

Further work supported the results of Brown and co-workers, though failed to address the pathogenic significance of the expression. McMillan et al showed that mRNA levels for MMP-9 were elevated in both occlusive and aneurysmal aortic tissue when compared to controls(McMillan et al., 1995a). Thompson and colleagues provided some of the first truly quantitative data regarding MMP-9 in aortic tissue, using ELISA to demonstrate a six and ten-fold increase in MMP-9 levels in occlusive disease and aneurysmal disease respectively. Furthermore, while TIMP-1 levels were increased in aneurysmal tissue, occlusive aorta showed similar levels to controls, suggesting an imbalance between enzyme and inhibitor levels(Thompson et al., 1995).

There has been similar interest in the contribution of MMP-2 in more mature atherosclerotic lesions. Li and colleagues used a combination of immunostaining, zymography and Western blotting to demonstrate an increase in the level and expression of MMP-2 in human atherosclerotic aortic tissue compared to non-diseased tissue(Li et al., 1996). They demonstrated increased MMP-2 activity and co-localised this to areas of macrophage infiltration, suggesting a possible role for MMP-2 in the development of the acute complications of atherosclerosis. As with the previous reports, this study was limited by the sample size of only 12 cases. There were problems with the methodology, using tissue collected from autopsies of trauma victims who were young, with no history of cardiovascular disease.

The Role of MT1-MMP

Since the discovery of MT1-MMP and its role in the activation of MMP-2, the question arose as to whether plaque MT1-MMP expression may be raised in parallel with the expression of MMP-2. This question was addressed recently by a study in which the expression and activity of MT1-MMP was examined in normal and atherosclerotic arteries by a combination of immunohistochemistry, Northern blotting, Western blotting and gelatin zymography(Rajavashisth et al., 1999b). In normal vessels, MT1-MMP expression was detected in medial smooth muscle cells and to a lesser extent in the adventitia. In atheroma, MT1-MMP expression was found to co-localise with macrophages and smooth muscle cells. Cultured smooth muscle cells and macrophages were exposed to pro-inflammatory cytokines

IL-1 α and TNF- α plus oxidised LDL. TNF- α stimulated a 3.8 fold increase in macrophage expression of MT1-MMP within 6 hours, whilst IL-1 α , TNF- α and oxidised LDL exposure produced a 2-4 fold increase in expression and protein level by cultured smooth muscle cells in a time dependant manner.

They concluded that cytokine stimulation of macrophages and smooth muscle cells within plaques may upregulate MT1-MMP expression, influencing the activation of MMP-2 and ecm re-modelling. However, they did not provide evidence of a quantitative increase in enzyme level in atheroma nor activity, and while the response to cytokines in vitro was clear, the situation within the plaque in vivo is clearly more complex.

Further work has demonstrated the expression of other MMPs within atherosclerotic tissue, including the presence of MMP-7 and MMP-12 in human carotid lesions retrieved at endarterectomy(Halpert et al., 1996).

Evidence of Increased Proteolysis

The mere presence and expression of MMPs within plaques does not necessarily confer an increased risk of plaque instability. However, an increase in proteolytic activity, or an imbalance between the levels of enzymes and their inhibitors, may lead to an overall increase in matrix degradation. Difficulty had arisen with this regard since the preparation of tissue extracts in which MMPs are separated from their substrate and inhibitors prior to assay activates the gelatinases, preventing a true assessment of the in vivo state(Vine and Powell, 1991).

Evidence an imbalance between MMPs and TIMPs has been provided by Knox and co-workers, who studied proteolytic activity in aortic tissue from 25 patients with occlusive or aneurysmal disease and compared it to 7 normal aortas(Knox et al., 1997). They performed immunohistochemical staining for MMP-1,-2 and -3, TIMP-1 and TIMP-2, and employed a technique of in situ zymography to identify a net increase in proteolytic activity in excess of endogenous inhibition(Galis et al., 1995). This was a semi-quantitative process in which frozen sections of tissue were incubated upon fluorescently labelled substrate, areas of proteolysis appearing as dark lytic zones. Compared to normal aortic tissue, both occlusive and aneurysmal aorta demonstrated a marked increase in staining for MMP-1 and MMP-3 plus a

smaller increase in TIMP-1. MMP-2 was also increased in the absence of an increase in TIMP-2. Whilst normal tissue failed to exhibit proteolytic activity, both disease subgroups demonstrated gelatinolytic and caseinolytic activity on their luminal aspect. There were limitations of the in situ zymography technique, such as the inability to control for variability in specimen proteoglycan concentration or provide a substrate truly characteristic of the extra cellular matrix. That aside, the study did provide evidence of an imbalance between MMPs and their inhibitors, leading to a net increase in overall proteolysis.

The expression of TIMPs is likely to provide protection against plaque instability. When atherosclerotic lesions were compared to non-diseased tissue, TIMP-3 expression was fivefold higher in the plaques (Fabunmi et al., 1998). This was co-localised to macrophages in the vulnerable areas of the plaque, in particular the cap and shoulder of the plaque, but also to a lesser extent medial smooth muscle cells. In contrast to isolated monocytes in vitro, macrophages expressed TIMP-3. Cultured vascular smooth muscle cells also expressed TIMP-3 along with TIMPs -1 and -2, and this was up regulated by the addition of PDGF and TGF- β in the absence of cellular proliferation. This contradicted the imbalance theory, though more work is required to study the specific role of TIMP-3. In particular, no data was provided in this study about histological features of instability nor patient symptomatology.

Further evidence for TIMPs counterbalancing the increase in MMP levels has been provided in rabbits (Zaltsman et al., 1999). Cholesterol fed animals developed atherosclerotic lesions that exhibited a 2-3 fold increase in TIMPs -1 and -2 levels expressed by endothelial cells, smooth muscle cells and macrophages. In situ zymography again demonstrated excess MMP activity but in the areas of increased TIMP production this was less evident. On the other hand, increased levels of TIMP-1 in mice have been shown to reduce the development of atherosclerotic lesions (Rouis et al., 1999). Apolipoprotein E-deficient mice, susceptible to developing accelerated atherosclerosis, subjected to adenovirus mediated over-expression of TIMP-1 demonstrated a significant decrease in the size of lesions despite identical lipid and lipoprotein profiles. There was also a significant decrease in macrophage infiltration, plus MMP-2, MMP-3 and MMP-13 levels. Dietary lipid lowering has also been shown to reduce MMP-1 activity and macrophage infiltration in the aortas of rabbits (Aikawa et al., 1998b). They also demonstrated a decrease in activity of MMPs -2, -3 and -9 in tandem with an increase in the collagen content of the vessel wall and increased accumulation of mature smooth muscle cells (Aikawa et al., 1998a).

Whilst these studies provided some useful insight into the pathogenesis of these lesions, extrapolation of animal data to the human scenario is difficult, since the nature of lesion development is probably quite different.

More recently attempts have been made to associate the observed increase in proteolytic activity with evidence of plaque instability. Sukhova provided evidence of increased collagenolysis in unstable carotid plaques (Sukhova et al., 1999). They compared MMP-1 and MMP-13 levels, based on immunohistochemistry and Western blotting, in 10 predominantly fibrous lesions with 10 atheromatous plaques. The levels of both enzymes were significantly higher in the atheromatous lesions. They also demonstrated co-localisation of collagenase-cleaved collagen with macrophages expressing MMP-1 and MMP-13, loss of type I collagen and increased collagenolysis. MMP-1 and MMP-13, the interstitial collagenases, are required for the initial step in collagen degradation, cleaving triple-helical fibrils of collagen types I, II and III at a single site (Mitchell et al., 1996), producing fragments which are accessible to other proteolytic enzymes, in particular the gelatinases and stromelysins. Whilst the collection of tissue specimens was not standardised and the classification of tissue perhaps oversimplified, the study did provide evidence of an association between enzyme levels and collagen degradation in plaques.

Evidence of an imbalance between enzymes and their inhibitors and the potential for excessive matrix degradation raises a number of other issues. Firstly, matrix degradation due to increased proteolysis preceded acute plaque disruption, a systemic factor which predicted those plaques at risk would be extremely valuable, so preventative intervention could be better targeted.

Work so far has focused on the localised nature of the imbalance but one study measured peripheral blood levels of the gelatinases in patients with coronary disease (Kai et al., 1998). The levels of MMP-2 and MMP-9 were measured by ELISA in 33 patients with acute coronary syndromes, 17 with stable angina and 17 normal control subjects. The level of MMP-2 in patients with stable angina was only slightly raised compared to controls, but those with acute coronary syndromes were twofold higher. This increase was sustained for 7 days after treatment. The level of MMP-9 was increased only in the acute coronary patients, twofold in patients with myocardial infarction and threefold in those with unstable angina, but the increase was only sustained for up to 3 days, returning to the control levels by day 7. These serial changes in blood enzyme levels may be useful in determining patients at risk, and are particularly interesting given the localised nature of plaque destabilisation.

Secondly, it is clear that certain individuals have susceptibility toward plaque de-stabilisation. Why such an imbalance should occur remains an enigma, particularly recognising that atherosclerosis is a multifactorial disease, combining genetic and environmental factors. As discussed previously, oxidised LDL is believed to play a crucial role in atherogenesis though the precise mechanism is unclear. Recent work has shown that human monocytes allowed to differentiate into macrophages in culture and exposed to oxidised LDL underwent an up-regulation in the expression of MMP-9(Xu et al., 1999). Interestingly, the expression of TIMP-1 was reduced, and the addition of HDL, known to be protective against atherosclerotic disease, abrogated the increase in MMP-9 expression. This provided a particularly attractive model for the events leading up to plaque disruption, whereby oxidised LDL within plaques may stimulate macrophage-mediated extracellular matrix degradation through increased expression of MMP-9, whilst down-regulating TIMP-1 expression.

Genetic Factors

Several genetic factors have been identified as increasing susceptibility to atherosclerosis. For example, several polymorphisms in the fibrinogen gene cluster are associated with differences in plasma fibrinogen, a strong risk factor for coronary artery disease(Green and Humphries, 1994). Since the interest regarding a role for MMPs in atherogenesis, it has been suggested that genetic variations controlling MMP expression may predispose individuals to an imbalance between proteolysis and extracellular matrix accumulation(Ye et al., 1998). Ye and colleagues have studied sequence variations in the MMP-3 gene, detecting a variant in the 5'-flanking region of the gene promoter, arising from an insertion/deletion of an adenosine 1171 bp upstream from the start of transcription(Ye et al., 1995). This resulted in a run of 6 adenosines (6A) in one allele and 5 adenosines (5A) in the other. 72 patients known to have coronary heart disease were genotyped and studied prospectively by angiography. The 6A6A genotype was significantly associated with an increased progression of coronary atherosclerosis, with the 5A/6A demonstrating a slower progression. They hypothesised that the weaker promoter led to a reduction in MMP-3 leading to a net increase in matrix accumulation and disease progression. Clearly this is in contrast to any potential role in acute plaque disruption where the balance is tipped the other way. Subsequent work in vitro showed that the 5A allele had a twofold higher promoter activity than the 6A allele and thus may lead to less extracellular matrix accumulation and tip the balance in favour of degradation(Ye et al., 1996). A study of

330 patients with myocardial infarction showed that the 5A/6A allele was detected more frequently than in a control population (48.8% versus 32.7% respectively, $p < 0.0001$) (Terashima et al., 1999). This was independent of all other cardiovascular risk factors, suggesting a possible genetic marker for increased susceptibility to plaque rupture.

More recently a polymorphism has been detected in the MMP-9 gene promoter, arising from a cytosine to thymidine transition at position -1562 relative to the start of transcription (Zhang et al., 1999). Transfection experiments and DNA-interaction assays showed that the thymidine allele had a higher promoter activity than the cytosine allele, probably related to the binding of a putative repressor protein to the cytosine residue. Genotype studies of 584 male patients following myocardial infarction showed a significant increase in the frequency of the thymidine allele in those patients with angiographic coronary stenoses of $>50\%$, suggesting an association between this polymorphism and severity of disease. Further work is required to validate these results and establish any potential role in plaque instability.

Summary

Other factors potentially involved in plaque development have been discussed, including infectious agents, heat shock proteins, lipoproteins and cytokines. Some of these have been shown to directly influence MMP expression and production while others seem to have a more tenuous and secondary association. Certainly the process is a complex one, but there is now substantial evidence of an imbalance between MMPs and their inhibitors forming the final step in plaque development leading to degradation and acute disruption. MMPs are also intricately involved in the process of aneurysm formation and intimal hyperplasia. What has been lacking in previous work has been true quantitative data relating to recognisable features of plaque instability, namely patient symptomatology and histological confirmation of disruption. The potential for enzyme imbalance causing plaque disruption raises the exciting possibility of therapeutic intervention to prevent such acute change.

CHAPTER FIVE

MMP INHIBITION

5.1 Introduction

5.2 TIMPs

5.3 Specific MMP Inhibition

Synthetic peptides

Doxycycline

Inhibition of MMP Synthesis

Other Agents

5.4 Summary

5.1 Introduction

The realisation that tissue remodelling due to increased MMP activity plays a key role in disease states has led to considerable interest in the potential for MMP inhibition. To date, most clinical and pre-clinical data regarding therapeutic manipulation of the extracellular matrix has been in the fields of arthritis, periodontal disease and cancer(Dollery et al., 1995). The hypotheses behind MMP inhibition are twofold; firstly that cellular migration is MMP dependent, inhibition trapping the cell in its matrix surround, and secondly that an imbalance between enzymes and inhibitors leads to excessive tissue degradation as seen in aneurysms and unstable plaques. In this case inhibition may redress the balance back in favour of matrix accumulation. Potential methods of inhibition include:

- 1) Increasing the level of TIMPs either by the exogenous administration of recombinant TIMPs or stimulating their local production through gene therapy.
- 2) Administration of synthetic inhibitors, either specifically targeting MMPs or using compounds which effect MMP activity by a secondary mechanism to their intended use.
- 3) Reducing the production of MMPs.

5.2 TIMPs

Work on TIMPs has been restricted to animal models and in vitro culture of human vascular cells. Both TIMP-1 and TIMP-2 have been shown to inhibit tumour growth and metastasis in animals(Alvarez et al., 1990). In vascular disease, as described previously, increased expression of TIMP-1 by rat smooth muscle cells reduced intimal hyperplasia(Dollery et al., 1999a), and the TIMP-1 deficient mouse developed significantly more neointima after arterial injury than that expressing TIMP-1(Lijnen and Collen, 1999). In vitro, adenovirus mediated gene transfer of the human TIMP-1 gene inhibited smooth muscle cell migration and neointimal formation(George et al., 1998). Local over-expression of TIMP-1 by transfection of TIMP-1 DNA to smooth muscle cells in a rat model of aneurysmal disease prevented rupture(Allaire et al., 1998) and increased levels of TIMP-1 in mice have been shown to reduce the development of atherosclerotic lesions(Rouis et al., 1999). Similarly in an animal model of atherosclerosis,

adenovirus mediated over-expression of TIMP-1 led to a significant decrease in the size of atheromatous lesions despite identical lipid and lipoprotein profiles(Rouis et al., 1999).

It is difficult to extrapolate these data to potential applications in humans. The major drawback associated with TIMPs would be tissue delivery, since exogenous products would be metabolised and denatured with minimal tissue penetration at the intended site of action. Treatment would have to take the form of local tissue delivery or gene therapy. Cytokine stimulation of TIMPs would probably have significant side effects precluding clinical use. Clearly either will be very expensive to develop, so most interest has been concentrated on the development of synthetic inhibitors.

5.3 Specific MMP Inhibition

Synthetic Peptides

A number of synthetic inhibitors have been investigated, in particular specific synthetic peptides, tetracycline based antibiotics and anthracyclines. Synthetic peptides are largely analogues of the cleavage site in the collagen molecule with a metal-binding group in the position of the cleaved peptide bond. The metal binding group, usually a thiol, sulphydryl, carboxyl or hydroxamate group, binds to the zinc atom in the activity site of the MMP(Schwartz et al., 1991).

One of the first synthetic peptides was Batimastat which has a broad spectrum inhibitory action against the MMPs via a direct toxic action. This showed promising results in prevention of tumour development and metastasis in animal models(Watson et al., 1996) and has been tested in clinical trials, though the long term use is precluded by its lack of oral bioavailability(Parsons et al., 1997). In aneurysmal disease Batimastat was shown to limit the expansion of abdominal aortic aneurysms(Bigatel et al., 1999). This study employed the rat model of elastase infusion-induced aneurysm formation, and daily treatment with Batimastat significantly reduced aortic dilatation compared to controls. Elastin preservation was observed accompanied by an attenuation of the inflammatory response.

Marimastat is a second-generation inhibitor with a similar structure and mode of action to Batimastat. Unlike Batimastat, Marimastat is active orally(Denis and Verweij, 1997).

Preliminary results in cancer patients were promising though concerns were raised about significant musculoskeletal side effects in 30% of patients(Talbot and Brown, 1996).

Marimastat has been studied in vascular models in vitro. Segments of human saphenous vein cultured with Marimastat demonstrated a reduction in neointimal thickening. A dose response relationship was observed, and an effect identified using a concentration of the drug in medium equivalent to the plasma levels obtainable at the dose used in clinical cancer trials(Porter et al., 1998). This was paralleled by a significant reduction in MMP-2 and MMP-9 activity. Similar results were seen following angioplasty in the rat, where administration of a systemic MMP inhibitor similar to marimastat reduced the early migration of smooth muscle cells(Bendeck et al., 1996). However, in this model there was a long term 'catch-up' phenomenon because of increased smooth muscle cell proliferation, and further work is required to clarify this in the human model.

In the in vitro aneurysm model, Marimastat prevented the elastase-induced degradation of elastin in aortic tissue in vitro in parallel with a reduction in the level of activated MMP-2(Treharne et al., 1999). Further hydroxamate-based MMP antagonists have been used to demonstrate suppression of experimental abdominal aortic aneurysms in the elastase-infusion rat model(Moore et al., 1999), and also in chronic flow mediated arterial enlargement after the formation of arterio-venous fistulae in rats(Karwowski et al., 1999).

Concerns about the long term use of such compounds in view of their relatively broad spectrum and potential side effects have led to the development of more specific inhibitors(Michaelides and Curtin, 1999), including orally acting specific inhibitors of MMP2 and MMP-9(Tamura et al., 1998). Results of their use, and other specific synthetic inhibitors, in models of vascular disease are awaited with interest.

Doxycycline

Doxycycline is a member of the tetracycline antibiotic family that, apart from its anti-bacterial action, is also a potent non-selective MMP inhibitor. Its main mode of action is through binding to the active zinc site, though it also binds to a non-active calcium site resulting in conformational change in the enzyme structure and loss of activity(Greenwald et al., 1998). It may also suppress MMP expression(Uitto et al., 1994). Coupled with their low cost, clinical

availability and good safety profile, the tetracyclines are an attractive option in studies of MMP inhibition.

Most work in clinical trials has been in the field of periodontal disease, where clinical improvement has been associated with a reduction in collagenase activity(Rifkin et al., 1993). Oral administration of doxycycline in patients with osteoarthritis before hip surgery reduced collagenase and gelatinase activity in extracts of human cartilage(Smith et al., 1998), confirming results from animal models(Yu et al., 1992). More recently it has also been shown to reduce malignant cell proliferation and MMP-1 activity in a canine model of osteosarcoma(Cakir and Hahn, 1999).

In vitro, doxycycline has been shown to prevent intimal hyperplasia using an established human vein culture model, by inhibition of MMP activity (Figure 5.1)(Loftus et al., 1999). It also prevented aneurysmal degeneration of the elastase-induced rat model of abdominal aortic aneurysm, causing elastin preservation associated with reduced MMP-9 activity(Petrinec et al., 1996). Similar results have been obtained using chemically modified tetracyclines without antibiotic activity, compounds which may have even fewer side effects than doxycycline and no potential for encouraging bacterial resistance in long term use(Curci et al., 1998a).

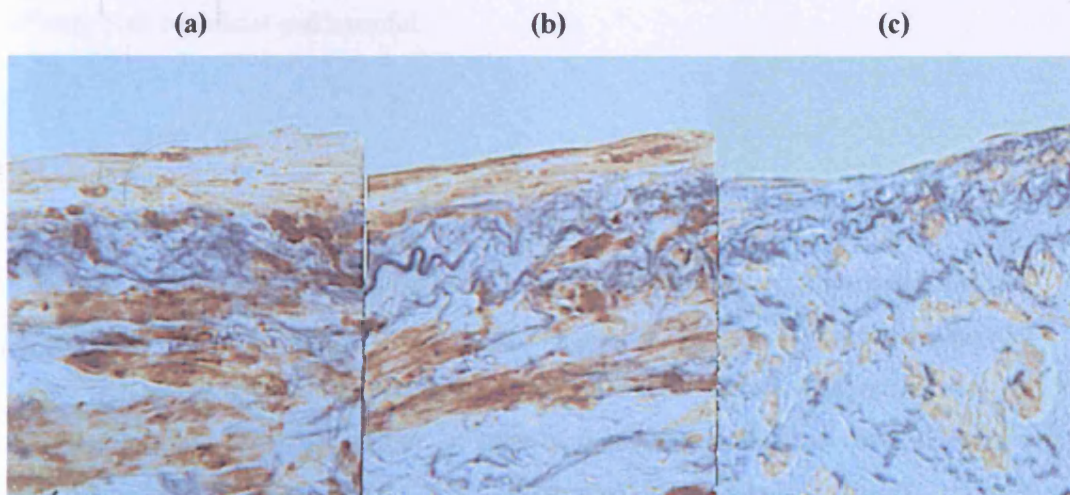


Figure 5.1: Doxycycline caused a dose response reduction in human venous intimal hyperplasia, in tandem with reductions in MMP activity (a= control, b and c represent increasing doses of doxycycline in culture).

In a human model of aneurysmal disease in vitro, doxycycline prevented elastin degradation(Boyle et al., 1998). Intravenous tetracycline given to patients prior to aneurysm

surgery showed rapid penetration of the arterial wall(Franklin et al., 1999b). More importantly, patients who receive treatment preoperatively for 7 days with oral low-dose doxycycline exhibited a three-fold reduction in the aortic wall expression of MMP-2 and a four-fold reduction in expression of MMP-9(Thompson and Baxter, 1999). On the basis of these results the authors called for a prospective randomised controlled trial to assess the effect of long term treatment on the expansion of small aneurysms.

Inhibition of Enzyme Synthesis

The inhibition of MMP synthesis can be approached from the conventional pharmaco-therapy and more experimental molecular standpoints. TGF- β , retinoids and corticosteroids have all been shown to down-regulate the expression of MMPs. However, their practical use in vivo is limited. For example, steroids have proved ineffective in preventing intimal hyperplasia, and though heparin has been shown to reduce expression of MMP-3 and MMP-9, it does not eliminate activity, nor prevent either atherosclerosis or intimal hyperplasia(Dollery et al., 1995). The alternative approach via application of antisense oligonucleotides to the MMP genes is possible but clinical application awaits much more detailed study of the potential effects, both beneficial and harmful.

Other Agents

Targeting therapy at an earlier stage in the proteolytic cascade may prevent side effects associated with generalised MMP inhibition, and plasmin has been suggested as one such target. Guinea pig to rat xenografts, seeded with rat smooth muscle cells retrovirally transduced with the rat plasminogen activator inhibitor-1 gene, failed to develop aneurysms, exhibiting preservation of arterial elastin(Allaire et al., 1998). Control animals, lacking the transduced gene but with the vector alone or no seeding at all, developed aneurysms, the majority of which ruptured.

Other groups have targeted the inflammatory response within aneurysms including the inhibition of prostaglandin E₂, which regulates the expression of MMP-9. Prostaglandin E₂ has adverse effects upon smooth muscle cell viability and cytokine secretion in vitro and indomethacin has been shown to reduce cytokine release and prostaglandin production in aortic

explants(Franklin et al., 1999a). Indomethacin, by reducing the synthesis of prostaglandin E2 also decreased the production of MMP-9 in the rat model of aneurysm development(Miralles et al., 1999). A case control study of patients taking non-steroidal anti-inflammatory drugs demonstrated a lower rate of aneurysm growth rate compared to controls, suggesting a potentially effective treatment for preventing aneurysm expansion(Walton et al., 1999). Other non-steroidal drugs have been shown to modulate MMP activity, though their action in vivo requires detailed clinical studies(Barracchini et al., 1998).

There has been considerable interest in the beneficial effect exerted by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors in patients with coronary and cerebrovascular disease(Furberg, 1999). A recent prospective study in patients with a history of myocardial infarction and elevated LDL cholesterol treated with fluvastatin demonstrated a 32% reduction in stroke risk(Plehn et al., 1999). Also, fluvastatin reduced the progression of angiographic coronary artery disease and event-free survival compared to placebo treatment(Ballantyne et al., 1999).

Initially this was thought to be purely secondary to a lipid lowering effect, but this now appears to be more complex(Hughes, 1996). Two members of the group, fluvastatin and simvastatin, have been shown to inhibit smooth muscle cell migration and proliferation independent of their lipid lowering activity(Corsini et al., 1995). Further work by the same group suggested a direct anti-proliferative effect upon the arterial wall(Corsini et al., 1998) and fluvastatin treatment in a rat model of intimal hyperplasia prevented neointima formation in this way(Soma et al., 1995).

In vitro work has shown that HMG CoA reductase inhibitors directly interfered with the adherence of monocytes to endothelium by reducing the expression of monocyte cell surface receptors, again independent of their lipid lowering effect(Weber et al., 1999). This has also been shown in parallel with a down-regulation in P-selectin expression on endothelial cells(Pruefer et al., 1999). Moreover, both fluvastatin and simvastatin inhibit macrophage secretion of MMP-9 in vitro(Bellosta et al., 1998).

The beneficial action of HMG CoA reductase inhibitors in patients with coronary and cerebrovascular disease seems to be multifactorial, with lipid lowering effects, but also direct actions on vessel wall cell-cell interactions and MMP production. They therefore offer the exciting potential for modification of matrix regulation in vascular disease states.

Summary

A broad spectrum of therapeutic agents have the potential to modify the proteolytic effect of MMPs in vascular disease, both at the level of expression and secretion, and also by reducing activity. Much of the evidence comes from in vitro and animal work, though some of these agents have been shown to be beneficial in human studies. Further work is required to clarify the nature of the benefit and potential long term side effects, particularly with tetracyclines and selective synthetic MMP inhibitors.

CHAPTER SIX

THE MANAGEMENT OF CAROTID DISEASE

6.1 Introduction

6.2 Investigation

Duplex Scanning Versus Angiography
Other Techniques

6.3 Treatment

The Role of Carotid Endarterectomy
Carotid Endarterectomy and the Prevention of Complications
The Role of Carotid Angioplasty

6.4 Summary

6.1 Introduction

Atherosclerotic carotid disease may present with a stroke, where loss of focal cerebral function persists for more than 24 hours, or transient ischaemic attack, defined as symptoms lasting less than 24 hours(Hatano, 1976). These can take the form of temporary loss of cerebral function or monocular visual loss (amaurosis fugax). Approximately 80% of ischaemic strokes are secondary to carotid disease(Naylor et al., 1998b). In some patients, carotid stenoses are detected in the absence of symptoms either by the detection of a bruit, or during investigation of an unrelated problem, particularly coronary disease. Internal carotid artery disease is often localised to the origin, particularly the posterolateral wall, probably because the area is exposed to low shear stress, flow stasis and flow separation(Zarins et al., 1983).

6.2 Investigation

Duplex Scanning Versus Angiography

Duplex ultrasound combines B-mode imaging with waveform analysis using pulsed wave Doppler, obtained by insonating the artery with an electronic cursor. Colour duplex uses a red or blue shade to demonstrate flow towards or away from the probe. This, combined with the waveform pattern of velocity against time, can accurately assess the degree of carotid stenosis in more than 95% of patients and in many units has replaced conventional angiography as the 'gold standard' investigation(Loftus et al., 1998).

Angiography is performed by direct femoral artery puncture under local anaesthetic, with images obtained in at least 2 planes. Digital subtraction angiography has improved the quality of the images obtained by conventional means (Figure 6.1). There has been considerable debate over the role of angiography in the assessment of carotid stenoses, largely related to the small but significant risk of complications. The overall risk of neurological complications is 0.5%, but this increases to 3.7% in symptomatic patients(Davies and Humphrey, 1993). Furthermore, in patients with a stenosis of >50%, the angiographic risk increases to 5% transient ischaemic complications and 4% permanent. This almost certainly reflects severity of disease and catheter induced cerebral embolisation, with the risk reduced by obtaining aortic arch views only, but to an extent compromising quality of images.



Figure 6.1: Carotid arteriogram revealing significant stenosis at the origin of the right internal carotid artery.

There is controversy over the accuracy of duplex at assessing the degree of stenosis, but this argument can be extended to angiography. Angiographically, there are 3 different methods, each measuring the diameter of maximum stenosis, but each comparing it to different measurements of non-diseased vessel. Considering the 2 major trials of carotid endarterectomy, the North American Symptomatic Carotid Endarterectomy Trial (NASCET) used the diameter of a segment of disease free artery above the stenosis, while in the European Carotid Surgery Trial (ECST), the normal arterial diameter was estimated from the stenosed segment. The ECST method tends to over-estimate the degree of narrowing. There is also relatively poor intra- and inter-observer variability in angiographic assessment. When stenoses are divided into groups relating to the percentage narrowing, intraobserver agreement is 83%, with interobserver agreement only 75%(Chikos et al., 1983).

For duplex scanning, there are also different criteria used for measuring severity of stenosis. Each unit needs to assess the accuracy of their criteria, since much depends upon operator experience and quality of equipment, as well as angiographic technique and method for

calculating stenosis. Using a combination of B-mode imaging plus a peak systolic velocity of >200 cm per second to diagnose stenoses of greater than 70% can reduce the angiography rate to under 5% without encountering unexpected findings at operation(Martin et al., 1995). Compared directly to angiography, the accuracy of such criteria is said to be more than 90%(Naylor et al., 1998b).

A further potential advantage of duplex scanning is the study of plaque morphology and prediction of those plaques at risk of acute disruption. The Gray-Weale classification divides plaques into 4 types according to the degree of echolucency (Gray-Weale, 1988). Unfortunately correlation both with histological features of instability and risk of thrombo-embolic events is variable(Reilly et al., 1983). It is possible to quantify the echolucency of the plaque using computerised analysis of the grey scale content of the ultrasound image of the plaque, and the grey scale median value has been correlated with the presence of cerebral infarction on CT scan and histological degree of lipid and haemorrhage content(Belcaro et al., 1996; El-Barghouty et al., 1996). However, there are problems with the standardisation of such a technique, and there is a lack of data to support a true correlation with increased risk of stroke or embolisation. The technique seems unlikely to be able to accurately predict those plaques most at risk, leading to interest in other methods of plaque characterisation.

Other Techniques

Intravascular ultrasound has been studied, predominantly in coronary disease, and although accurate in assessing degree of stenosis it appears to be limited in providing details about plaque composition and the propensity for disruption(Kleber et al., 1998). Angioscopy similarly has been used in coronary patients and provides an accurate detection of thrombus and plaque surface disruption, with correlation between angioscopic features and risk of acute myocardial infarction(Uchida et al., 1995). Both techniques are invasive and may have a significant risk of thrombo-embolic complications in patients with carotid disease. Optical coherence tomography is a new technique measuring the optical interference between light reflected from a sample and a reference mirror, providing much better resolution of plaques than ultrasound(Brezinski et al., 1996). Whilst this appears promising, further work is required to assess the ability to characterise plaques and correlate it with increased risk.

Thermography has been used to measure plaque temperature in both carotid and coronary arteries. In the carotid plaque, Casscells et al measured the temperature at 20 sites in each of 50 carotid plaques at endarterectomy using a thermistor(Casscells et al., 1996). Higher temperature correlated closely with the intensity of macrophage infiltration in the plaque. A similar technique found that the difference in temperature between coronary plaques and healthy vessel wall was higher in patients with unstable angina and acute myocardial infarction than stable angina(Stefanadis and Toutouzas, 1998). This raised the possibility that techniques such as infrared thermography may detect areas of high metabolic activity and identify plaques at risk of acute disruption.

Other techniques for measuring the degree of carotid stenosis include magnetic resonance angiography (MRA), high resolution magnetic resonance imaging and spiral CT angiography. MRA has been compared to both duplex and angiography, with an accuracy of 94% and 88% respectively. However, motion artefacts and claustrophobia prohibit its use in 10% of patients, and duplex remains a more cost-effective option(Turnipseed and Kennell, 1993). Spiral CT may be similarly restricted and requires further evaluation and comparison to duplex(Cumming and Morrow, 1994). High resolution MRI has been shown to be very accurate at determining the actual size of carotid lesions retrieved at endarterectomy(Yuan et al., 1998), and is also sensitive in the detection of intraplaque lipid and calcification, though not thrombus(Shinnar et al., 1999). It has not been compared directly with either duplex or angiography.

6.3 Treatment

The Role of Carotid Endarterectomy

Carotid surgery was first described in 1954 as a treatment for thrombo-embolic disease of the internal carotid artery(Eastcott et al., 1954). Its role in current vascular practice is based upon the results of 2 large international randomised controlled trials comparing results of operative intervention and optimal medical therapy, namely the NASCET and ECST(North American Symptomatic Carotid Endarterectomy Trial Collaborators, 1991; European Carotid Surgery Trialists Collaborative Group, 1991). Best medical therapy comprises optimising vascular risk factors such as hypertension and hyperlipidaemia, plus institution of anti-platelet therapy. Aspirin alone has been shown to reduce long term stroke risk by 25%(Antiplatelet Trialists Collaboration, 1988).

Carotid endarterectomy conferred a six-tenfold reduction in the long term risk of stroke compared to best medical therapy for patients with a symptomatic 70-99% stenosis on angiography, equating to an absolute and relative risk reduction of 10-15% and 44-54% respectively (Table 6.1). No benefit was observed in those patients with lesser stenoses.

	<i>ECST</i> (n=778)	<i>NASCET</i> (n=659)
Mean follow up	32 months	24 months
Best medical therapy		
All deaths/strokes at 1 month		3.3%
All deaths/disabling strokes at 1 month		0.9%
All deaths		0.3%
All strokes at end of study	22%	28%
Best medical therapy and CEA		
All deaths/strokes at 1 month	7.5%	5.8%
All deaths/disabling strokes at 1 month	3.7%	2.1%
All deaths	0.9%	0.6%
All strokes at end of study	12.3%	12.6%

Table 6.1: *Results of the ECST and NASCET trials for patients with >70% stenosis*

The role of carotid endarterectomy in patients with asymptomatic stenoses remains rather unclear. The Asymptomatic Carotid Atherosclerosis Study reported a 55% reduction in stroke risk, from 11 to 5.1% based on projected life table analysis to 5 years (Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, 1995). A larger scale trial, the European Asymptomatic Carotid Surgery Trial, continues to recruit patients and the results are awaited with interest (Nicolaidis, 1994), though it seems likely that large numbers will need to be treated to prevent relatively few strokes.

In patients with >70% stenosis, if the perioperative risk could be eliminated, endarterectomy would reduce the risk of stroke by 75%. It may alter the perceived benefit in patients with lesser stenoses and asymptomatic disease. This has led to the adoption of a number of quality control methods to minimise operative complications.

Carotid Endarterectomy and the Prevention of Complications

Carotid endarterectomy can be performed under either local or general anaesthetic. An incision is made along the anterior border of the sternomastoid muscle, with the carotid bifurcation identified after division of the common facial vein. Following mobilisation of the artery and systemic heparinisation, the artery is clamped distal to the disease. The common carotid and external carotid arteries are also clamped, and a longitudinal arteriotomy performed. A shunt can then be inserted to maintain cerebral perfusion, and the plaque is then dissected away carefully securing the distal end with tacking sutures. The arteriotomy is closed primarily or as a patch angioplasty using vein or prosthetic material as shown in Figure 6.2.

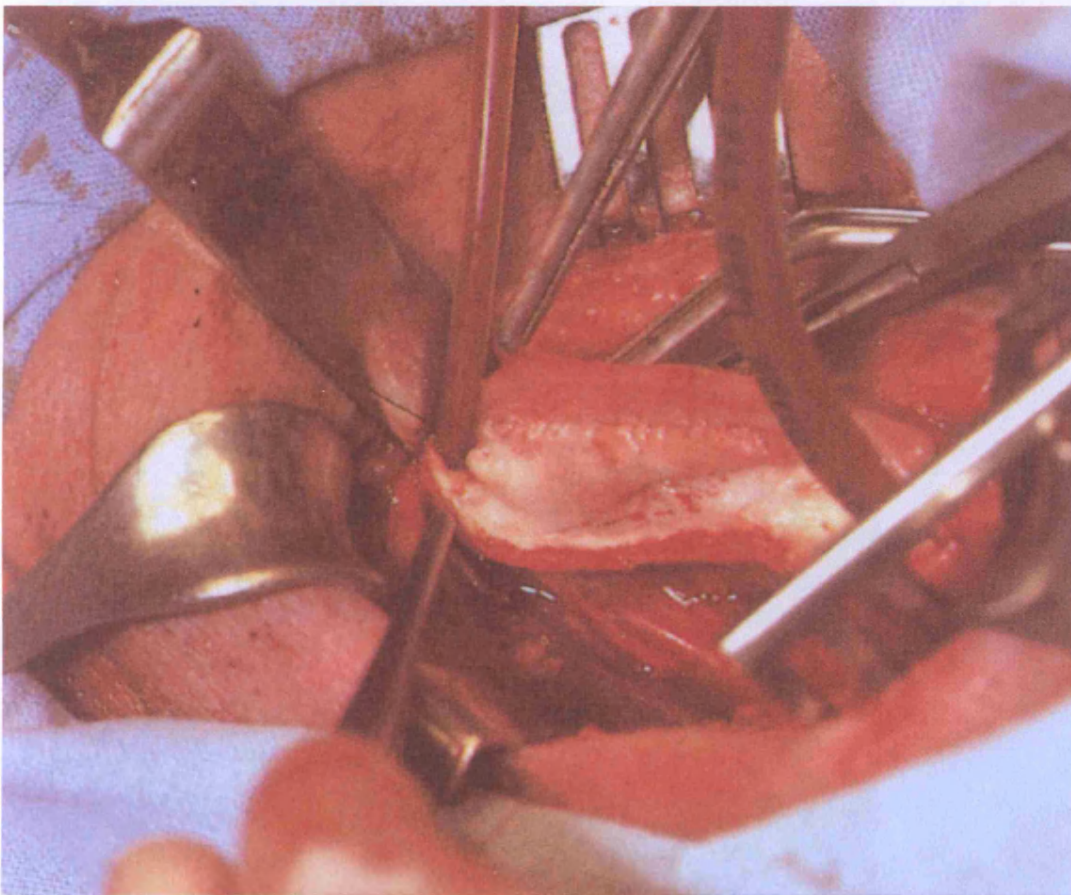


Figure 6.2: *Closure of the endarterectomised internal carotid artery with a dacron patch.*

Postoperative blood pressure control is essential since hypertension, which affects up to 66% of patients, can lead to myocardial infarction and cerebral hyper-perfusion (Naylor and Ruckley, 1996). Meticulous surgical technique can prevent nerve injuries and reduce wound

complications. However, the most feared complication is perioperative stroke, with intraoperative outnumbering postoperative strokes. Intraoperative strokes are usually of embolic or cerebral hypo-perfusion origin. The use of transcranial Doppler (TCD) monitoring of the middle cerebral artery blood flow velocity can detect problems with cerebral perfusion allowing adjustment of the shunt, and also particulate emboli which may direct the surgeon towards altering surgical technique, such as early clamping (Smith et al., 1998). TCD employs a low frequency (2.5 MHz) pulsed ultrasound wave beam directed through the temporal bone (Figures 6.3). The detection of intraoperative embolisation has been shown to correlate with a reduction in cognitive function (Gaunt et al., 1997). It is also useful in the postoperative period at detecting embolisation that may be a sign of impending carotid thrombosis. Early detection can lead to urgent re-exploration or the instigation of dextran therapy, shown to reduce embolisation and the risk of neurological events (Lennard et al., 1999).

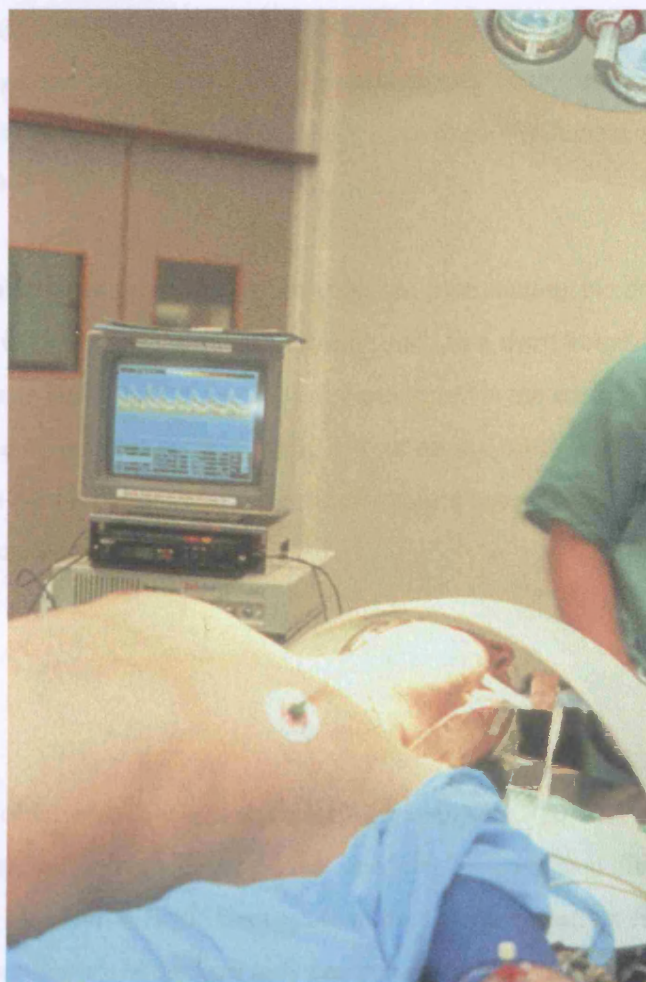


Figure 6.3: Patient being monitored preoperatively with transcranial Doppler.

Other methods of quality control include the use of intraoperative arteriography, ultrasound or angioscopy to detect technical error. A rigid policy of quality control has been shown to reduce the perioperative stroke risk to less than 2%(Naylor and Gaunt, 1996; Lennard et al., 1999).

The Role of Carotid Angioplasty

The success of percutaneous transluminal balloon angioplasty in the treatment of peripheral vascular disease has led to interest in its potential role in carotid disease. A number of studies have suggested that it is feasible but currently only one randomised-controlled trial has been published(Naylor et al., 1998a). This trial was suspended when 5 of the 7 patients who underwent angioplasty suffered a stroke. This was probably caused by the angioplasty procedure dislodging particulate matter causing emboli, the numbers of which were 8 times higher during angioplasty than endarterectomy as detected by TCD(Jordan et al., 1999). The results of other trials such as the CAVATAS trial are awaited(McGuinness and Burnand, 1996; Sivaguru et al., 1996).

There are considerable potential benefits of angioplasty, plus stenting in combination with cerebral protection techniques, over endarterectomy, such as a short hospital stay, less wound or nerve complications and access to more distal disease within the internal carotid artery. However, there is no evidence as yet to support its routine use, and with the low complication rate now associated with endarterectomy, the disadvantages in terms of embolic stroke may outweigh the potential benefits.

6.4 Summary

The role of carotid endarterectomy in symptomatic patients with >70% internal carotid artery stenoses is now clear, and good quality control procedures have reduced the major complication rates. However, it is still impossible to predict which patients are particularly at risk since some lesser degrees of stenosis may become unstable or develop rapidly. The option of pharmacotherapy remains an attractive one, both to prevent acute plaque disruption and to decelerate progression of early disease.

SECTION TWO: METHODS

CHAPTER SEVEN

PATIENTS AND METHODOLOGY

7.1 Patients

Patient Recruitment

Patient Demographics

Preoperative Imaging and Emboli Detection

7.2 Sample Procurement and Enzyme Extraction

Procurement of Tissue Samples

Plasma Samples

MMP Extraction

7.3 MMP Quantification

Enzymography- Principles

Enzymography- Methods

Enzymography- Quantification

Immunoblotting

Enzyme Linked Immuno-sorbent Assay

7.4 Histological Analysis and Immunostaining

7.5 Studies of MMP Expression

Messenger RNA Extraction

Reverse-Transcription Polymerase Chain Reaction

In Situ Hybridisation

7.6 Statistical Analysis

7.1 Patients

Patient Recruitment

Seventy five consecutive patients admitted for carotid endarterectomy (CEA) to the Leicester Royal Infirmary were entered into this study between September 1996 and April 1997. Local ethics committee approval was obtained for the procurement of specimens and all patients gave full informed consent for the study.

A clinical history was obtained from each patient, with particular care taken to establish the number and duration of ischaemic events, together with a record of the time between the last symptom and the operation. A record was made of all past medical problems, particularly risk factors for cardiovascular disease such as smoking history, diabetes, hypertension, ischaemic heart disease and hyperlipidaemia. Medications including antihypertensives, antiplatelet agents and HMG CoA reductase inhibitors were also documented. All patients underwent a thorough neurological examination to assess for residual neurological deficits.

Patient Demographics

The median age of the patient cohort was 68.5 years, with a range from 46 to 84 years. There was a slight preponderance of males compared to females and the majority admitted to a past or present history of smoking. As expected, more than 50% had a history of hypertension, and a similar proportion had a history of ischaemic heart disease, either in the form of angina or a previous myocardial infarction. There were relatively few diabetics or patients with hyperlipidaemia.

Each patient was assigned preoperatively to one of 4 symptom groups on the basis of their symptom free duration. Group 1 (N=20) consisted of all asymptomatic patients (either with severe bilateral disease or as part of the Asymptomatic Carotid Surgery Trial) and Group 2 (N=16) those with symptoms more than 6 months preoperatively. Group 3 (N=18) comprised those patients with symptoms less than 6 months but more than 1 month preoperatively, and Group 4 (N=21) all those with symptoms in the month prior to surgery. The latter included some with frequent recent cerebral ischaemic episodes.

Focal cerebral ischaemic events were defined as transient ischaemic attack, amaurosis fugax, central retinal artery occlusion or cerebrovascular accident. Those patients with vague symptoms such as dizziness and headaches, but an absence of definite focal cerebral events were assigned to Group 1.

Of the symptomatic patients, 7 presented with amaurosis fugax and 29 with transient ischaemic attacks. Thirteen patients had suffered a stroke, 5 with residual neurological deficits, and 7 patients had a combination of presenting neurological events.

All patients were on long term aspirin therapy (75 or 150 mg daily), and there was no difference between the groups in the use of other medications including lipid-lowering agents or in serum lipid levels between the groups. There was no significant difference between the major demographic features or cardiovascular risk factors between these four groups (X^2 test, $p>0.05$) (Table 7.1).

Preoperative Imaging and Emboli Detection

Although patients undergo duplex scanning by an experienced vascular technologist in the outpatient setting to assess suitability for surgery, all patients underwent a further pre-operative duplex ultrasound assessment of the carotid plaque for quantification of the degree of stenosis and to exclude occlusions. A diagnosis of a severe internal carotid stenosis ($>70\%$) was based on a combination of B mode imaging and a peak systolic velocity of $>200\text{cm/s}$ $\pm$ end diastolic velocity $>110\text{cm/s}$. These criteria have been validated within the unit and by others with an accuracy of 93-94% (Loftus et al., 1998). All of the patients in this study had a 70% or greater internal carotid artery stenosis, with no difference between the groups in the percentage stenosis (Kruskal-Wallis test, $p>0.05$).

No patients underwent carotid angiography, nor did patients routinely undergo preoperative CT brain scans. We have found that CT is unhelpful unless specifically indicated (Gaunt et al., 1996b) and carotid angiograms are only performed if the ultrasonographer performing the preoperative duplex scan is concerned about proximal or distal disease, sub-occlusion of the internal carotid artery or abnormal vascular anatomy.

Risk Factor	Symptom Group			
	1 n=20	2 n=16	3 n=18	4 n=21
Age (median/range years)	68.5 (46-81)	68.5 (53-82)	68.5 (46-82)	68 (49-84)
Sex (male:female)	15:5	6:10	12:6	12:9
Smoking	16 (80%)	10 (62%)	14 (78%)	18 (86%)
IHD	12 (60%)	7 (44%)	9 (50%)	13 (62%)
Hypertension	12 (60%)	11 (69%)	11 (61%)	13 (62%)
Diabetes	2 (10%)	2 (12%)	5 (28%)	5 (24%)
Hyperlipidaemia	5 (25%)	2 (12%)	4 (22%)	4 (19%)

Table 7.1: *Clinical risk factors for patients assigned to each of four symptom groups on the basis of symptom free duration.*

Definitions: Smoking = past or present history of smoking;
IHD= ischaemic heart disease (past history of myocardial infarction/CABG/angina requiring treatment);
hypertension = high blood pressure requiring treatment;
diabetes = insulin and non-insulin dependant diabetes requiring treatment including diet controlled;
hyperlipidaemia based on current medication and/or random fasting lipid levels.

There was no significant difference between the groups for any of the independent risk factors.

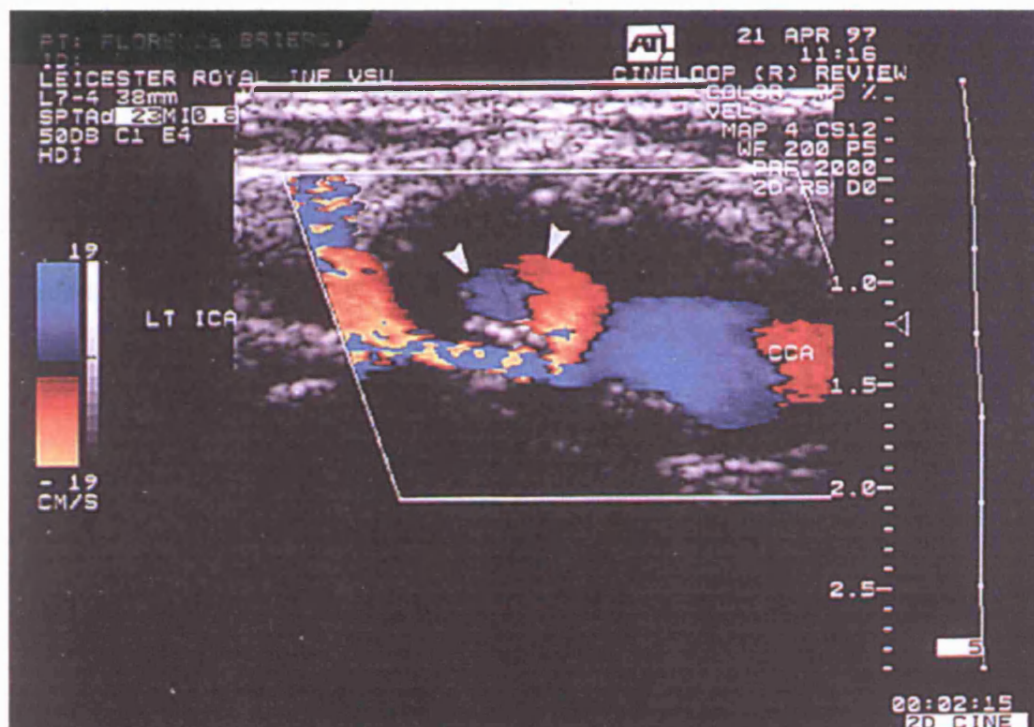


Figure 7.1: *Carotid Duplex Scan.*

Preoperatively patients were monitored with trans-cranial Doppler (TCD) for 30 minutes, plus intraoperatively during the dissection phase of the operation. This aimed to identify those with ongoing particulate micro-embolisation, highly indicative of plaque instability (Gaunt et al., 1996b). Continuous TCD monitoring of the ipsilateral middle cerebral artery was performed using a SciMed PC Dop 842 TCD (SciMed, Bristol, UK). Signals were recorded onto digital audiotape for off line analysis and interpretation of embolic signals using an established model of emboli quantification (Figure 7.2). Previous work in Leicester has defined an accurate method for the quantification of particulate emboli (Smith et al., 1995).

Prior to this work quantification of embolic phenomena had proved difficult, in particular distinguishing between air and particulate emboli, largely because of amplitude overload and poor time resolution. Smith and co-workers re-routed embolic signals away from the audio frequency amplitude and substituted the Wigner distribution function for the fast Fourier transform to improve time and frequency resolution. This enabled accurate determination of

emboli duration and velocity, the product of which is the sample volume length. This measurement represents the physical distance over which an embolic signal can be detected. The hypothesis in this study was that air reflected more ultrasound and would therefore be detected over a greater sample volume length. Comparison between the sample volume length of air and particulate emboli in vitro and in vivo led to the development of objective physical criteria upon which emboli characterisation is now based and employed routinely. Essentially air emboli had a sample volume length 10 times higher than particulate emboli (2 cm and 0.2 cm respectively). Any emboli with a sample volume length of less than 0.5 cm were recorded as particulate.

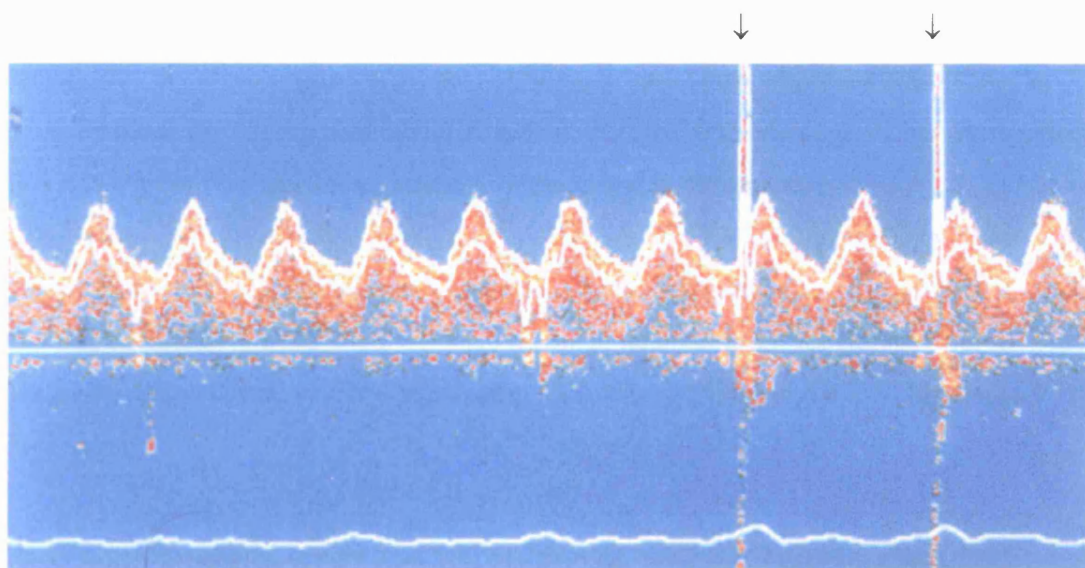


Figure 7.2: *Particulate emboli detected by TCD (↓)*

For the purpose of this study, emboli were only recorded if they occurred in the preoperative period or the dissection phase of the operation and, after internal carotid artery clamping, recording was discontinued.

7.2 Sample Procurement and Enzyme Extraction

Procurement of tissue specimens

Carotid plaques were obtained directly following endarterectomy. All operations were performed using standard surgical techniques and with minimal manipulation of the specimen. The endarterectomy was extended in a caudal direction to include a sample of non-diseased common carotid artery proximal to the plaque, but in continuity with the plaque, to act as a negative paired control. This did not involve a significant change to the standard operative technique.

During surgery, the presence of gross ulceration and plaque haemorrhage was noted and recorded by the surgeon with the plaque remaining in situ. Gross ulceration was defined as plaque surface irregularity seen by the surgeon at the time of arteriotomy. Plaque haemorrhage was noted grossly by looking for evidence of blood within the plaque.

The plaque and control tissues were divided longitudinally through the most apparent lesion or the area of tightest stenosis. Half was snap frozen in liquid nitrogen for storage prior to homogenisation. The other half was placed in fresh 4% paraformaldehyde for histological processing.

Plasma Samples

Blood samples were drawn from the peripheral vein of each patient 24 hours preoperatively. For the preparation of plasma, 2Na-EDTA (final 0.1%) was added to whole blood. Following centrifugation, plasma was siphoned into freezing vials, snap frozen in liquid nitrogen and stored at -80°C prior to blinded batch analysis of MMP levels by enzyme linked immunosorbent assay (ELISA).

MMP Extraction

The tissue was snap frozen in liquid nitrogen and stored at -70°C until extraction using the method of Vine and Powell (Vine and Powell, 1991). This extracts all forms of the enzyme,

either in the form of soluble proteins, vesicle entrapped proteins or proteins bound to the extracellular matrix.

Tissue was thawed on ice, weighed and roughly chopped into 1mm³ pieces which were placed in Du Pont tubes (Du Pont, USA). The tissue was agitated with phosphate-buffered saline (7.2g of NaCl, 1.48g of Na₂HPO₄·H₂O/l and 0.43g of KH₂PO₄/l) at 4°C to remove traces of haemoglobin. The tissue was then homogenised with 10mls/gram net weight tissue of homogenising buffer containing phenyl-methyl-sulphonyl fluoride (0.1mmol/l, Sigma, Poole, UK- see Appendix 1), using a polytron homogeniser (Kinematica, Switzerland). The resulting homogenate was centrifuged at 10 000g for one hour at 4°C. The supernatant was aspirated following centrifugation and transferred to small sections of visking tubing with a 12-14kDa molecular weight cut-off (Fisons, Loughborough, UK). The homogenate was dialysed for 18 hours at 4°C in immersed in dialysing buffer. The samples were sonicated (Soniprep 150, Sanyo UK Ltd) for 20 seconds at 5 microns to disrupt any protein aggregations.

The total protein concentration in each sample was estimated by the dye binding technique. Alterations in the absorbance of light at 595nm as measured by a spectrophotometer (UV-1601, Shimadzu Corporation, Japan) was associated with the dye (Biorad Laboratories) binding to protein molecules. Known concentrations of bovine serum albumin were used to construct a standard curve from which protein concentrations could be read.

The protein concentration was standardised for each sample to 0.9mg/ml with phosphate buffered saline, within the linear range for densitometric quantification. The compositions of all reagents are described in Appendix 1.

7.3 MMP Quantification

MMP levels were quantified by a combination of zymography and quantitative Enzyme Linked Immuno-sorbent Assay (ELISA). While casein and gelatin zymography are only semi-quantitative, they differentiate between active and latent enzyme forms. ELISA more accurately quantifies the enzyme level in a sample, but in some cases this reflects only the latent pro-enzyme form.

Enzymography-Principles

SDS-PAGE electrophoresis is a well described technique for separating proteins according to molecular size. Sodium dodecyl sulphate or SDS, a negatively charged detergent, is incorporated into a gel matrix and electrophoresis buffers denatures proteins within the specimen, SDS binding to positively charged proteins in proportion to the size of the molecule. Inclusion of a matrix substrate within the gel allows the detection of MMPs with certain specificities, in particular casein, which is degraded by collagenases, and denatured collagen, which is degraded by gelatinases. Gel electrophoresis results in elution of the proteins according to size.

After electrophoresis, the separated proteins are renatured by washing in Triton X-100 detergent, which displaces the SDS and activates the latent forms of the enzymes such that both active and latent forms degrade the gel substrate.

Enzymography-Methods

Proteins with gelatinolytic and collagenolytic activity were identified using substrate separating gels prepared by incorporating type III gelatin or casein respectively (Sigma, Poole, UK) into a 10% SDS-polyacrilamide gel (see appendix 1).

Type III collagen from calf skin, or casein was denatured by dissolving 10 mg in 4.0 ml of double distilled water, and heating in a water bath for an hour. The substrate was incorporated into the 10% polyacrilamide separating gel (Appendix I) during its casting, to a final concentration of 1 mg/ml. The 4% polyacrilamide stacking gel (Appendix I) was then cast with a 15-well comb (Biorad).

Once the gel was set, 15µl of each sample were mixed at room temperature with an equal volume of 2x non-reducing sample buffer (Appendix I) before application to the wells of the stacking gel. A positive control sample of conditioned media from HT-1080 cells, a human fibrosarcoma cell line known to constitutively express MMPs (obtained from the European Collection of Animal Cell Cultures, ECAEE No. 85111505) was included and a 'Multimark' multi-coloured standard ladder (15µl, Novex, San Diego, CA) consisting of myosin (250 kDa), phosphorylase B (148 kDa), glutamic dehydrogenase (60 kDa) and carbonic anhydrase (42

kDa) was also loaded.

Gels were electrophoresed in the BioRad protean II gel system in electrode running buffer at 4°C and 80mAmps for 2 hours. On completion of the run, the fractionated proteins were renatured by extensive washing in a solution of 2.5% Triton X-100 (Sigma, Poole, UK) on a rotary shaker at ambient temperature. Gels were then incubated for a further 18 hours at 37°C with gentle agitation in incubation buffer. At the end of this period gels were stained by transferring to Coomassie Brilliant Blue R250 (Sigma, Poole, UK) protein stain dissolved in a mixture of 50% methanol, 20% acetic acid and 30% double distilled water for 24 hours.

Bands of proteolytic activity are visualised as regions of clear lysis against the blue background of the gel. By comparing the relative migration of the bands and the molecular markers an estimation of the size of the proteases may be made. The degree of lysis correlates with the protease activity (Figure 7.3).

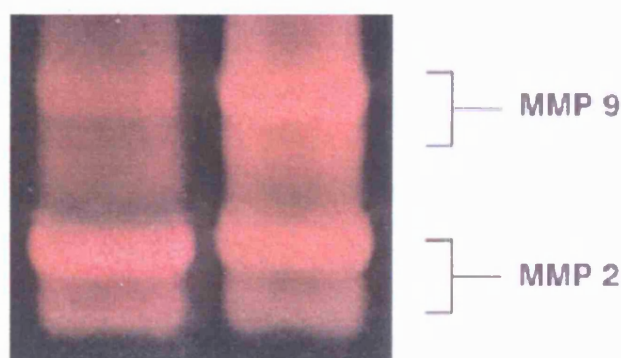


Figure 7.3: Clear bands against a blue background were identified as the active and latent forms of MMP-2 and MMP-9.

Enzymography-Quantification

The relative density of each lytic band was determined from negative photographic images of gels with a Pharmacia LKB Imagemaster scanning densitometer (Pharmacia LKB, St Albans, Herts, UK). The product of the optical density and area of the band was compared directly with the standardised positive HT-1080 control, to obtain a ratio comparable between gels and proportional to the amount of enzyme present. This method of quantification has been validated previously (Zucker et al., 1994).

The identity of the lytic zones on the gelatin zymograms were confirmed as MMP-2 and MMP-9 (72 kDa and 92kDa respectively) by immunoblotting

Immunoblotting

Western blotting allows identification of specific protein species which have been fractionated using gel electrophoresis. The separated proteins are transferred to a nitrocellulose membrane, to which antibodies are applied in conjunction with a suitable detection complex to located protein-antibody complexes. Correlation with molecular markers allows estimation of the size of detected species. Immunoblot analysis was utilised primarily to detect the presence of MMP-2 and MMP-9. Monoclonal antibodies raised in mice against human MMP-2 and MMP-9 (Oncogene, ,Camb., MA) were used

The stored frozen samples were thawed on ice and 200 µl aliquots were normalised for protein concentration as before (0.9 ng/ml). Both the SDS 10% polyacrilamide separating gel and SDS 4% stacking gel were assembled as before. However, the separating gel was cast without the incorporation of the gelatin substrate. The chosen samples were loaded along with the multi-coloured ladder and HT-1080 positive control, and the gels were run under non-reducing conditions as described above.

The proteins separated in the gel were then transferred electrophoretically to nitrocellulose membrane (ECL, Amersham, Bucks, UK) at 0.11 amps for 14 hours. Non-specific binding sites were blocked by immersing the membrane in 5% blocking buffer (Appendix I) for one hour at room temperature on a rotary shaker. The filter was then rinsed using two changes of washing buffer and then washed once for 15 minutes and twice for 5 minutes with fresh changes of the buffer at room temperature.

The membrane was then incubated in primary monoclonal antibody (diluted 1:1000 with blocking buffer) for one hour at room temperature. The membrane was then washed three times as before, and then incubated with a 1:2000 diluted horseradish peroxidase labelled anti-mouse secondary antibody (Amersham, UK) at room temperature. The membrane was then washed for fifteen minutes and then a further four times for five minutes each with fresh changes of washing buffer to minimise the background staining.

The following steps were then undertaken in a dark room. The excess buffer was drained from the washed membranes and placed on a piece of SaranWrap (Dow Chemicals), protein side up. Equal volumes of detection solution 1 (ECL, Amersham, Bucks, UK) were mixed to cover the membrane for one minute at room temperature. The ECL (Enhanced Chemiluminescence) detection system employs the emission of visible light (428 nm) following the cleavage of a luminal substrate by the horseradish peroxidase enzyme conjugated to the primary antibody. Luminal is oxidised to an excited state from which it decays to ground state via a light emitting pathway, which may be detected by exposure to blue light sensitive autoradiography film. The excess detection reagent was drained off and the membranes wrapped in SaranWrap, with air pockets smoothed out. The membrane was laid protein side up in a film cassette (Siemens) and a sheet of autoradiography film (Kodak X-OMAT-AR, Rochester, NY, US) was carefully placed on top of the membrane and exposed for varying lengths of time. The film was then developed using a Curix 60 developer (AGFA-Gevaert, Middlesex, UK).

Enzyme Linked Immuno-sorbent Assay

Further quantification was performed using ELISA techniques for MMP-1, MMP-2, MMP-3, MMP-9 TIMP-1, TIMP-2 and TIMP-1/MMP-1 complex using Biotrak assay systems (Amersham, Amersham, Bucks, UK), validated for use with human tissue homogenates (Fujimoto et al., 1993). These provide a specific and precise quantitative determination of enzyme levels and are based on a 2-site ELISA sandwich format as shown in Figure 7.4. They employ 2 antibodies directed against different epitopes of the enzyme.

During the first incubation step, MMP- present in the tissue homogenate binds to a microtitre plate precoated with antibody. During the second incubation step, detection antibody coated with horseradish peroxidase (HP) is added, which forms an immobilised complex. The amount of peroxidase bound to each well is determined by the addition of tetramethylbenzidine (TMB). The reaction is stopped by the addition of an acid solution and the resultant colour measured at 450nm in a microtitre plate spectrophotometer. The concentration of enzyme is determined by interpolation from a standard curve.

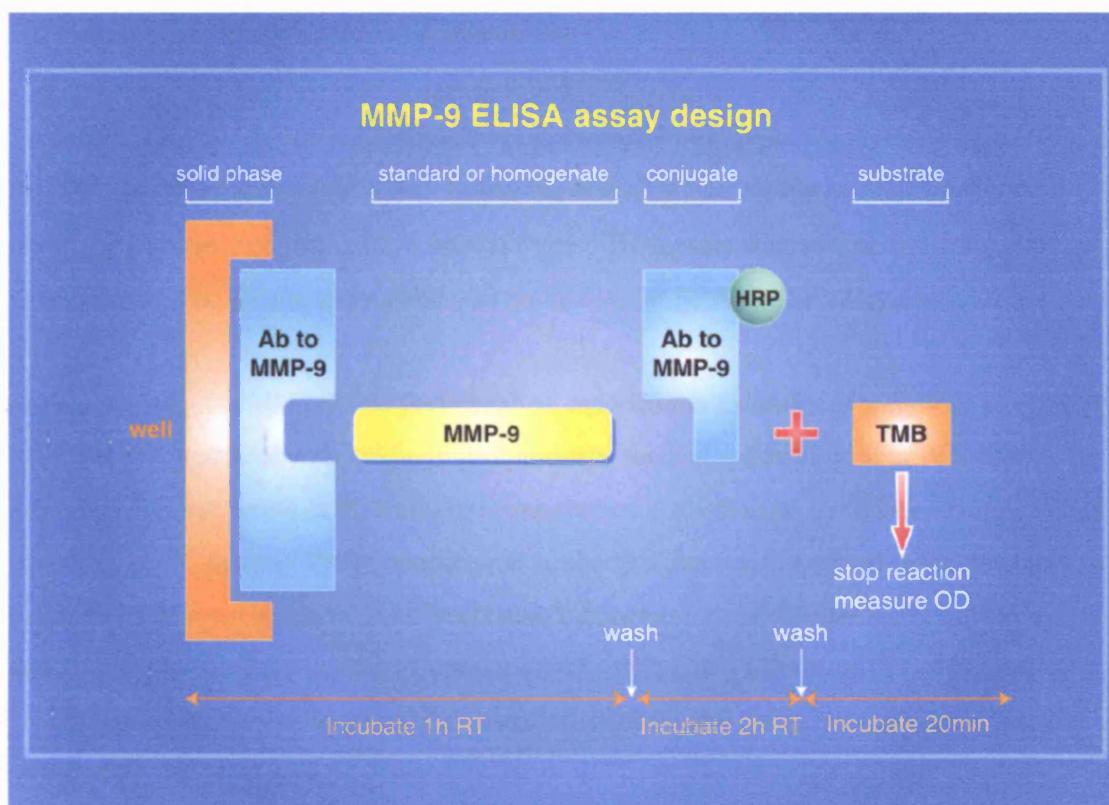


Figure 7.4: ELISA technique for the quantification of MMP-9 levels.

The MMP-2 and MMP-9 assays recognise free and TIMP complexed pro-enzyme only, showing no cross reactivity with activated forms of the enzyme nor other MMPs. The MMP-1 and MMP-3 assays recognise total enzyme both active and latent, plus enzyme complexed with TIMPs. The TIMP-1/MMP-1 complex assay recognises activated MMP-1 that has subsequently been complexed with TIMP-1, but not free MMP-1 or TIMP-1. The TIMP-1 assay recognises all TIMP-1, both free and complexed with MMPs, while the TIMP-2 assay detects all TIMP-2 except that bound to pro-MMP-2.

ELISA techniques have the ability to accurately quantify the level of total enzyme product in a specimen. They are unable to differentiate between the active and latent forms of the enzyme, thus give no impression of the overall level of proteolysis. Combined with zymography, however, they give an accurate assessment of the levels of active and latent forms.

7.4 Histological Analysis and Immunostaining

Immediately following removal, the section of plaque for histological analysis was placed in fresh 4% paraformaldehyde solution. Following overnight decalcification the samples were paraffin embedded and sectioned at 4µm intervals. These were stained with haematoxylin and eosin, Elastic Van Gieson and monoclonal antibodies for MMP-9 (R&D Systems, UK).

Immunostaining for MMP-9 was performed on deparaffinized sections. These were blocked for 20 minutes in methanol with 2% hydrogen peroxide, washed and incubated in PBS then incubated in horse serum in PBS at room temperature. After draining, sections were incubated overnight at 4°C in anti-MMP-9 monoclonal antibody. After washing the slides were incubated sequentially at room temperature in Vectastain biotinylated secondary antibody, ABC reagent and DAB substrate prior to nuclear counterstaining with Gill's Haematoxylin. After further washes the sections were dehydrated with graded alcohols and cleared in xylene.

Sections were evaluated by an experienced histopathologist (Dr Louise Jones, Senior Lecturer in the Department of Pathology, Leicester University), blinded to the clinical findings and identity of each patient. Four sections from each plaque were examined for the presence of plaque rupture, plaque cap thinning, intraplaque haemorrhage, intraplaque fibrosis, core necrosis, cap foam cells and graded for the degree of staining for MMP-9. Plaques were also classified as necrotic, fibrous or calcific on the basis of the predominant component of the plaque as previously described by Carr et al (Carr et al., 1996).

7.5 Studies of MMP Expression

Messenger RNA Extraction

Insufficient plaque material prohibited the division into 3 equal sections, thus for a further subset of 35 plaques, RNA was extracted from one half of the sample whilst the other half was prepared for MMP quantification as described. RNA extraction was performed using TRIzol total RNA isolation reagent (Life Technologies).

Sections of plaque were diced and placed in Du Pont tubes (Du Pont, USA), then homogenised in 1ml of TRIzol reagent per 100mg of tissue using the Polytron power

homogeniser. The samples were incubated for 5 minutes at 20°C to permit complete dissociation of nucleoprotein complexes. Chloroform (0.2ml/1ml of TRIzol) was added to the homogenates, the tubes shaken vigorously, then incubated at 20°C for 2 minutes. The samples were centrifuged at 12,000g for 15 minutes. This separates the mixture into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase in which the RNA remains exclusively.

The colourless upper aqueous phase containing all of the RNA was transferred to a fresh tube and the RNA precipitated by the addition of isopropyl alcohol (0.5ml/1ml of TRIzol). Following further incubation for 10 minutes at 20°C and centrifugation for 10 minutes at 12,000g, the RNA forms a pellet in the bottom of the tubes. The RNA pellet was washed thoroughly, resuspended in Rnase free water and snap frozen for subsequent analysis.

Reverse-transcription polymerase chain reaction

Reverse-transcription polymerase chain reaction (RT-PCR) was performed to confirm the expression of MMP-9. The reverse transcription reaction transcribes mRNA into copy-DNA (cDNA). The quality of the cDNA depends on the integrity of the mRNA and the fidelity of the transcription. The cDNA template is synthesised from RNA by extension of an annealed primer. This can be carried out using random primers, specific primers or by oligo (dT) priming.

Reverse transcription was carried out using AMV-RT enzyme and Oligo-dT₆ primers (Promega) as directed in the enzyme literature. Each reaction was made up of 5µl AMV reaction buffer, 4µl 10mM dNTPs, 2µl oligo dT₆ primer, 1µl RNAsin, 25ng total RNA, 10U AMV-RT enzyme, and the total volume made up to 25µl with DEPC-water. Negative control reactions lacked either template RNA or reverse transcriptase. All reactions were overlaid with mineral oil and incubated at 42°C for 60 minutes.

Amplification of specific sequences was performed using standard PCR methodology and primers designed according to sequences obtained from the GenBank database (sense 5'-aaggatccgactatgacaccgaccgtcg, antisense 5'-aagaattcggcgccggtagggtgta). PCR reactions were made up of 5µl PCR buffer (Appendix I), 1µl of each primer (10µM), 5µl RT product, 1µl Taq polymerase (Promega) and made up to a total volume of 50µl with DEPC-water.

Negative controls lacked cDNA template or Taq polymerase. A positive control was included, using a primer set for the GAPDH gene.

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

One disadvantage of maintaining the mRNA attached to oligo (dT) beads is that the precise amounts of mRNA cannot be measured spectrophotometrically. This is not a problem with cell culture techniques because the approximate mRNA yield can be calculated from knowledge of cell numbers, but with tissue sections it renders the technique non-quantitative. The method was therefore used to prove expression within the carotid plaque but no attempt was made to quantify this further.

In Situ Hybridisation

As with RT-PCR, in situ-hybridisation relies on the re-annealing of complementary sequences of nucleic acid. It differs from RT-PCR in that the nucleic acid can be identified in its original cellular site. A detailed cellular image is obtained using complementary hybridisation probes to that of the target sequence. Once a complimentary probe to the target has been made it is labelled, with digoxigenin for example, to allow visualisation.

For MMP-9, a well-established non-isotopic RNA in-situ hybridisation technique was performed using digoxigenin labelled oligonucleotide probes based on published sequences. The oligonucleotide sequences for probe synthesis were as follows:

```
actggcagggtttcccatcagcattgccgt
tccggcactgaggaatgatctaagcccagc
gttgaggcatcgtccaccggactcaaagg
gctccccctgccctcagagaatcgccagta
gcggctcctcaaagaccgagtcagcttgc
```


Histological sections were deparaffinised, rinsed in 2xSSC, and incubated with 100µl proteinase K (2µl/ml) for 60 minutes at 37°C. After washing the slides samples were pre-hybridised using 50µl of prehybridisation solution and incubated for 1 hour at 37°C. Digoxigenin-11-dUTP labelled probes were added to each pre-hybridised slide in 50µl of fresh pre-hybridisation solution and left at 37°C overnight.

The slides were then washed in 2xSSC/30% formamide twice, followed by incubation in filtered blocking solution for 10 minutes. Tissue sections were then incubated in anti-digoxigenin alkaline phosphatase, washed twice in TBS, then incubated in substrate buffer for 5 minutes. Subsequently each slide was incubated in the dark in 200µl substrate containing 8µl/ml nitro blue tetrazolium, 8µl/ml BCIP and 1µl/ml levamisole. Slides were checked microscopically until maximum signal occurred before background developed, then washed and mounted in aqueous mountant.

7.5 Statistical Analysis

All results are expressed as median values and inter-quartile ranges. Statistical analysis was performed using the SPSS statistical package (SPSS 8.0 for Windows, SPSS Inc., USA). Risk factors and individual histological features were analysed using the chi-squared test, whilst densitometry and ELISA results were compared using the Kruskal Wallis analysis of variance test. Differences in MMP levels between histological features and emboli detection were analysed using the non-paired, non-parametric Mann Whitney U-Test. Significance was assumed with a p value <0.05.

SECTION THREE: RESULTS

CHAPTER EIGHT

RESULTS

Histology and MMP Levels Based on Symptom Groups

8.1 Features of Plaque Instability

Macroscopic Features

Microscopic Features

Spontaneous Particulate Embolisation

8.2 MMP Quantification Based on Symptom Groups

Gelatin Zymography

-MMP-9

-MMP-2

MMP Levels Detected by ELISA

-Collagenases

-Gelatinases

-Correlation Between Zymography and ELISA

-The TIMPs

-MMP-1/TIMP-1 Complex

Summary of ELISA and Zymography

8.3 MMP/TIMP-1 Ratios- Evidence for an Imbalance Towards Proteolysis

8.4 Summary

8.1 Features of Plaque Instability

The macroscopic and microscopic appearance of the plaque was qualified in all cases and the results are shown in Table 8.1.

Macroscopic Features

Of the 75 plaques, 23 had macroscopic evidence of plaque rupture and only 9 had evidence of intraplaque haemorrhage (Figures 8.1 and 8.2). Although there was an increase in the proportion of plaques with macroscopic evidence of ulceration in the most recently symptomatic group, this failed to reach statistical significance. There was no difference in the incidence of macroscopic intraplaque haemorrhage.



Figure 8.1: Gross ulceration of the plaque retrieved from a patient with recent transient ischaemic attacks and spontaneous embolisation.

Feature	Symptom Group			
	1 (n=20)	2 (n=16)	3 (n=18)	4 (n=21)
Plaque rupture	4 (20%)	2 (12%)	3 (17%)	11 (52%)
Intraplaque haemorrhage	5 (25%)	3 (12%)	5 (19%)	12 (57%)
Plaque cap thinning	11 (55%)	9 (56%)	12 (67%)	14 (67%)
Intraplaque fibrin	7 (35%)	9 (56%)	12 (67%)	12 (58%)
Plaque necrosis	10 (50%)	10 (62%)	12 (67%)	16 (76%)
Cap foam cells	14 (70%)	8 (50%)	11 (61%)	16 (76%)
Macroscopic Ulceration	5 (25%)	3 (12%)	5 (19%)	10 (48%)
Macroscopic Haemorrhage	3 (15%)	1 (6%)	2 (11%)	3 (14%)
Emboli positive	2 (10%)	3 (12%)	4 (22%)	12 (57%)

Table 8.1: Features of plaque instability for each of the 4 symptom groups.

The increase in incidence of microscopic rupture and intraplaque haemorrhage in symptom group 4 was significant (χ^2 p-value =0.04 and 0.05 for respectively). The increase in spontaneous embolisation was also significant ($P=0.01$). None of the other features were significantly different between the groups.

Microscopic Features

Plaque cap thinning and evidence of plaque necrosis was common, in 50% or more of plaques for each symptom group. There was no difference between the groups. Similar results were obtained for the presence of cap foam cells and intraplaque fibrin. These were not quantified further.



Figure 8.2: *Plaque with evidence of intraplaque haemorrhage and cap thinning.*

Only 2 microscopic features were found to be significantly more common in the most recently symptomatic plaques, namely plaque rupture and intraplaque haemorrhage. Plaque rupture was identified microscopically in 52% of plaques in group 4 compared to 20% or less for each of the other 4 groups ($p < 0.05$). A similar result was observed for intraplaque haemorrhage with 57% in group 4 compared to 25% or less for each of the other groups. These results are similar to those reported previously regarding carotid plaque morphology. Van Damme and co-workers showed that 53% of complicated plaques were symptomatic and Carr presented a clear association between symptomatology and plaque rupture (Van Damme et al., 1994; Carr et al., 1996).

Plaques were classified as either predominantly fibrous or predominantly necrotic. Predominantly necrotic plaques were significantly more likely to demonstrate other features of plaque instability, in particular plaque rupture, intraplaque haemorrhage and intraplaque fibrin. This is demonstrated in Table 8.2. Representative histological sections are shown in Figures 8.3-8.5.

Microscopic Feature	Type of Plaque		χ^2 p-value
	Fibrous (n=41)	Necrotic (n=34)	
Plaque rupture	2	18	<0.001
Intraplaque haemorrhage	4	22	<0.001
Intraplaque fibrin	11	30	<0.001
Cap thinning	24	23	Ns
Cap foam cells	27	25	Ns

Table 8.2: *The incidence of microscopic features when plaques were classified by their predominant morphological feature.*

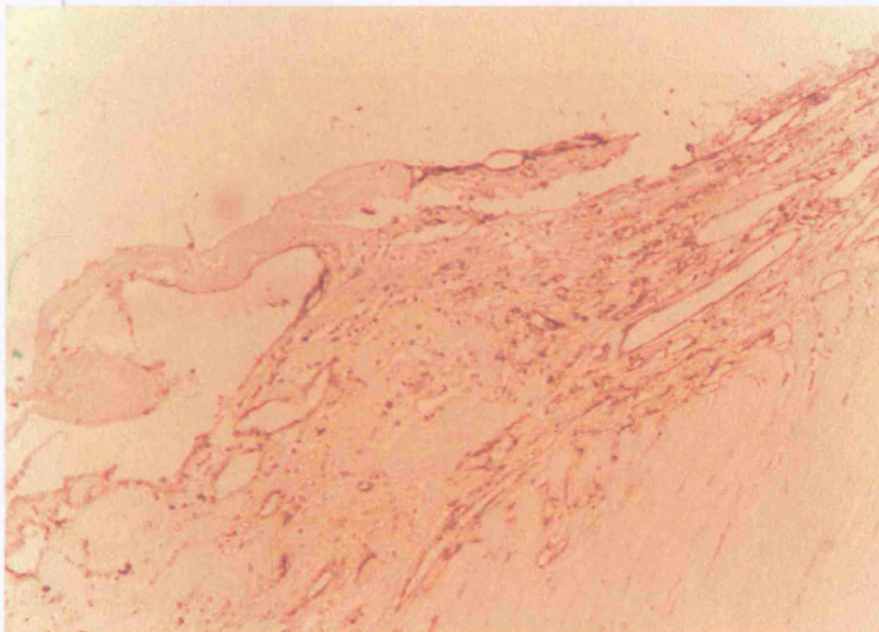


Figure 8.3: *Intense macrophage infiltration and foam cells in a plaque with evidence of cap thinning (x50 magnification, CD68 immunostain).*

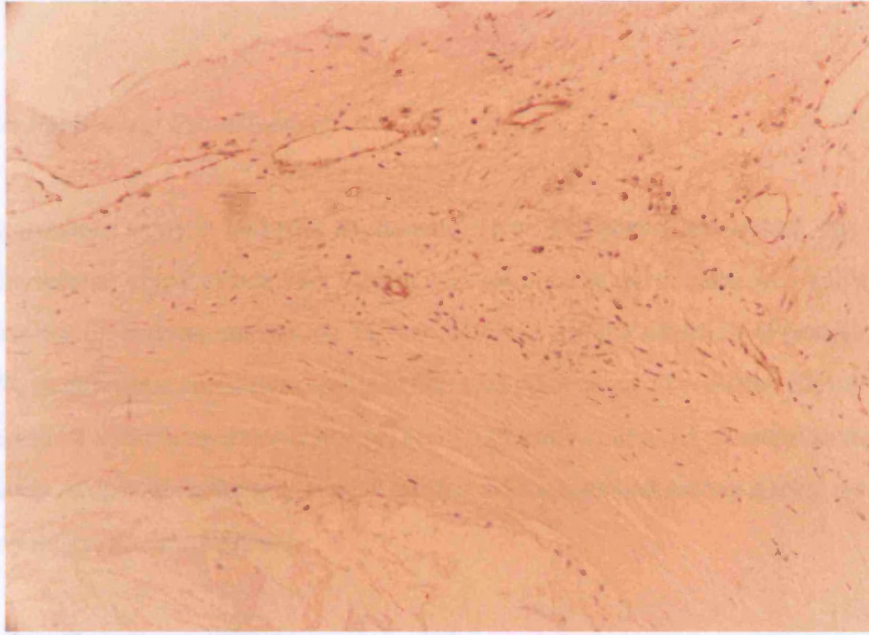


Figure 8.4: *Histological section through the shoulder of a plaque with an extensive necrotic core (x50 magnification, CD68 immunostain).*

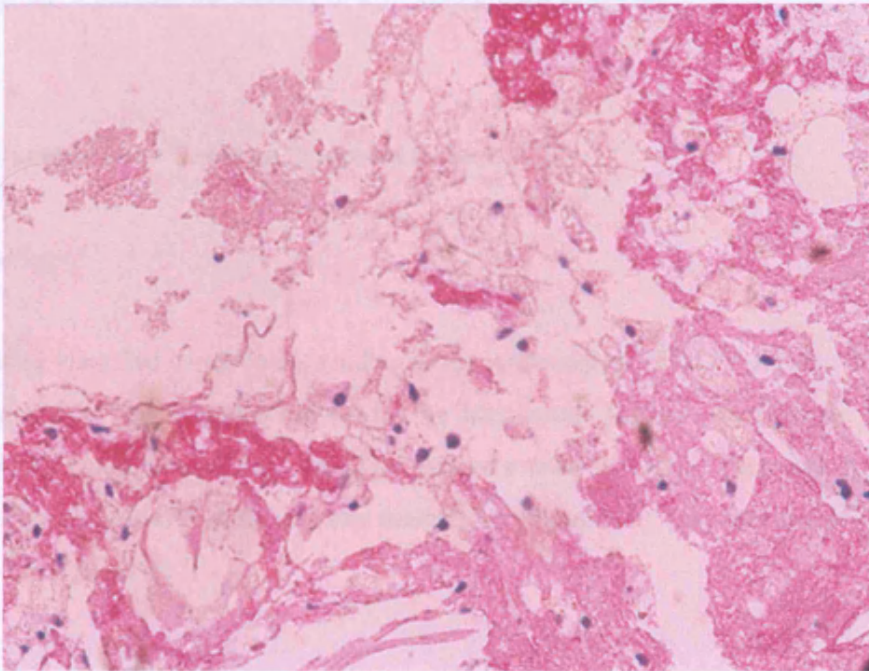


Figure 8.5: *Histological section through a carotid plaque demonstrating plaque fissuring (x100 magnification, H&E stain).*

Spontaneous Particulate Embolisation

Based on the previous study in Leicester to characterise air and particulate emboli, any signal with a sample volume length of less than 0.5 cm was recorded as particulate. In 5 patients there were no transcranial windows, preventing TCD monitoring and the detection of emboli. Of the remaining 70, spontaneous particulate embolisation was detected in 21 patients. Eleven patients were detected to be embolising preoperatively, and 10 of these continued to embolise during the dissection phase of carotid endarterectomy. A further 10 patients had emboli during the procedure but no preoperative emboli.

There was a significant increase in the number of plaques embolising in group 4 when compared to the other groups (12 in group 4 compared to 2, 3 and 4 in groups 1-3 respectively, $p=0.01$). Detection of spontaneous particulate embolisation in the cerebral circulation is probably the most sensitive indicator of plaque instability, identifying plaques during the acute process of thrombosis on the disrupted plaque surface. The significant increase in embolisation detected in group 4 justifies the banding of symptom groups in the study.

8.2 MMP Quantification Based on Symptom Groups

Immunoblotting

Immunoblotting identified bands corresponding to the inactive form of MMP-2 and MMP-9 as shown in Figure 8.6. The technique allowed for the detection of, but not quantification of the levels of, the individual proteins, because of the lack of standardisation and controls. It therefore merely confirmed that the enzymes detected by zymography (see later in this chapter) were MMP-2 and MMP-9.

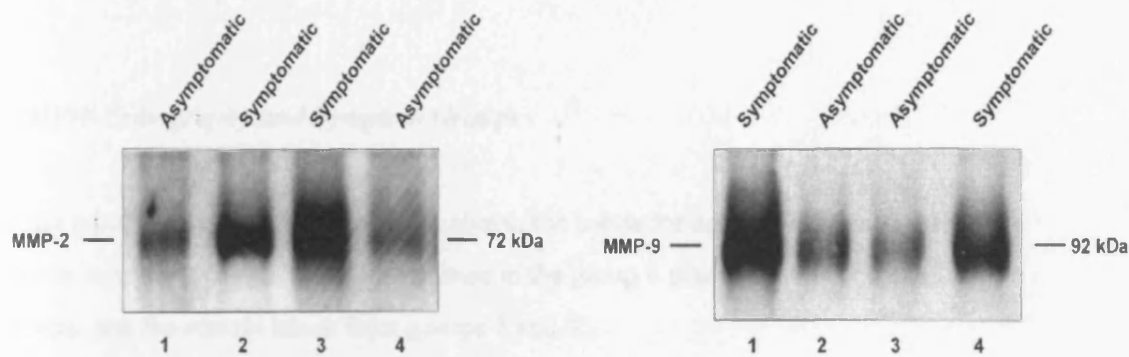


Figure 8.6: Immunoblotting confirmed the presence of MMP-2 and MMP-9 within carotid plaques

Gelatin Zymography

Negligible MMP activity was detected by casein zymography, but gelatin zymography detected 4 bands corresponding to the active and latent forms of MMP-2 and MMP-9 as shown in Figure 8.7.

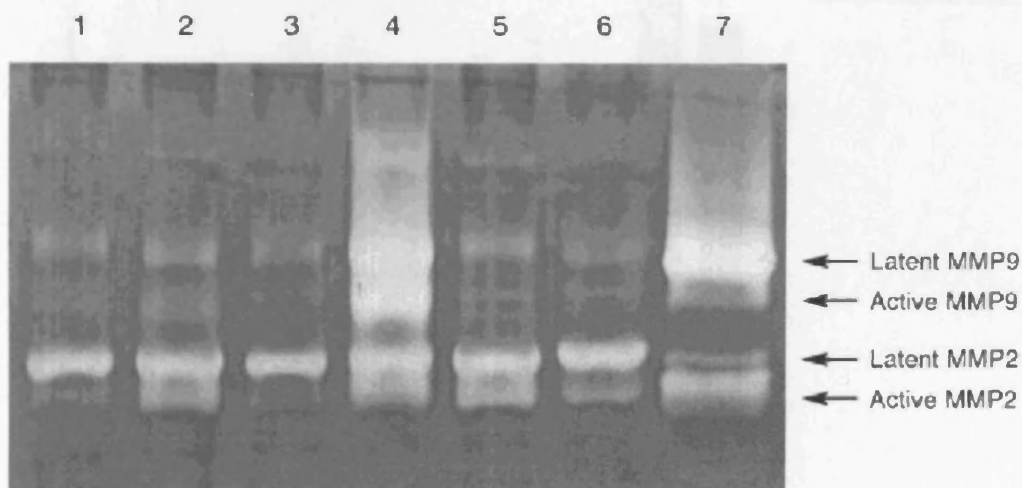


Figure 8.7: Representative gelatin zymogram showing 4 bands corresponding to the active and latent forms of MMP-2 and MMP-9. The lanes represent samples as follows:

Lanes 1-4: plaque tissue from symptom groups 1-4 respectively

Lanes 5-6: control tissue from groups 1 and 4 respectively

Lane 7: HT 1080 conditioned media control

MMP-9 Zymography and Symptom Groups

In the representative zymogram shown above, the bands for active and latent MMP-9 are clearly larger and the staining more intense in the group 4 plaque than the other symptom groups, and the control tissue from groups 1 and 4.

This was confirmed when quantified by densitometric analysis. Comparison to the HT 1080 control revealed that the densitometric ratio for group 4 approached double that of the other groups for both the active and inactive forms of MMP-9. This increase was found to be statistically significant ($p=0.001$ for both, Kruskal Wallis, Figure 8.8). This is the first time that the level of an active MMP has been shown to be quantitatively higher on the basis of symptomatology.

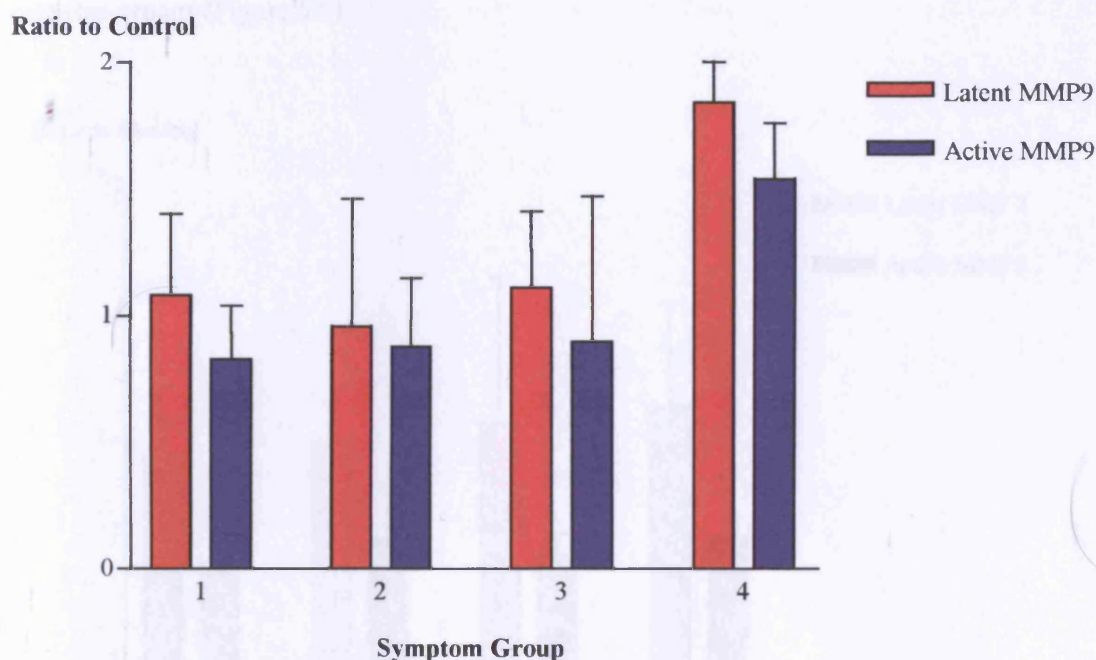


Figure 8.8: Bar graph showing the increase in both forms of MMP-9 as determined by densitometric analysis in the most recent symptom group compared to the other 3 groups (median and interquartile range, $p=0.001$ for both active and latent MMP-9).

MMP-9 Zymography: Plaques versus Controls

The levels of active and latent MMP-9 were significantly higher in the plaques of all symptom groups compared to the non-diseased control vascular tissue. This was particularly significant for the active form of the enzyme, which was barely detectable in control tissue ($p < 0.001$ for all groups). Latent enzyme was detectable in most cases but was significantly reduced compared to plaque levels ($p = 0.02$ for all groups). In contradistinction to the plaque MMP-9 levels, there were no differences in the MMP-9 levels measured in the control vascular tissue between the symptom groups. This suggested that the significant increase in MMP-9 activity within symptomatic plaques was a localised phenomenon

MMP-2 Zymography

The size and intensity of the bands representing both the latent and active forms of MMP-2 were found to be remarkably constant and there were no significant differences between the symptom groups (Figure 8.9).

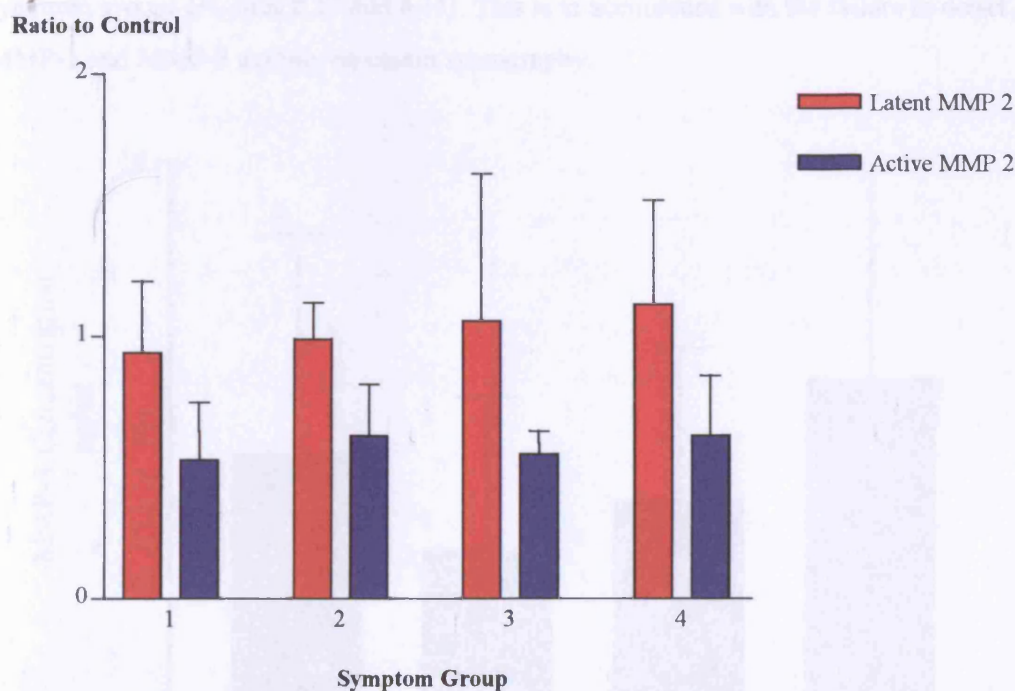


Figure 8.9: Bar graph showing the level of latent and active MMP-2 within the plaques on the basis of symptom group, as determined by densitometric analysis (median and interquartile range).

Nor was there a difference between MMP-2 levels in the plaques when compared to the MMP-2 levels measured in the control vascular tissue.

MMP Levels Detected by ELISA

As discussed previously, zymography is useful for measuring MMP activity, and in particular the analysis of proportionate levels of active and latent forms of the enzyme. However, densitometric analysis remains semi-quantitative, despite standardising for protein content and the introduction of a constant positive control. ELISA is the only truly quantitative measure of enzyme levels within tissue specimens.

ELISA: The Collagenases

The plaque levels of both MMP-1 and MMP-3 detected by ELISA were very low, ranging from 0-30 and 0-12 ng/ml respectively. These levels were almost identical to the levels detected in the control vascular tissue. There were no differences in either enzyme level between the symptom groups (Figures 8.10 and 8.11). This is in accordance with the failure to detect MMP-1 and MMP-3 activity on casein zymography.

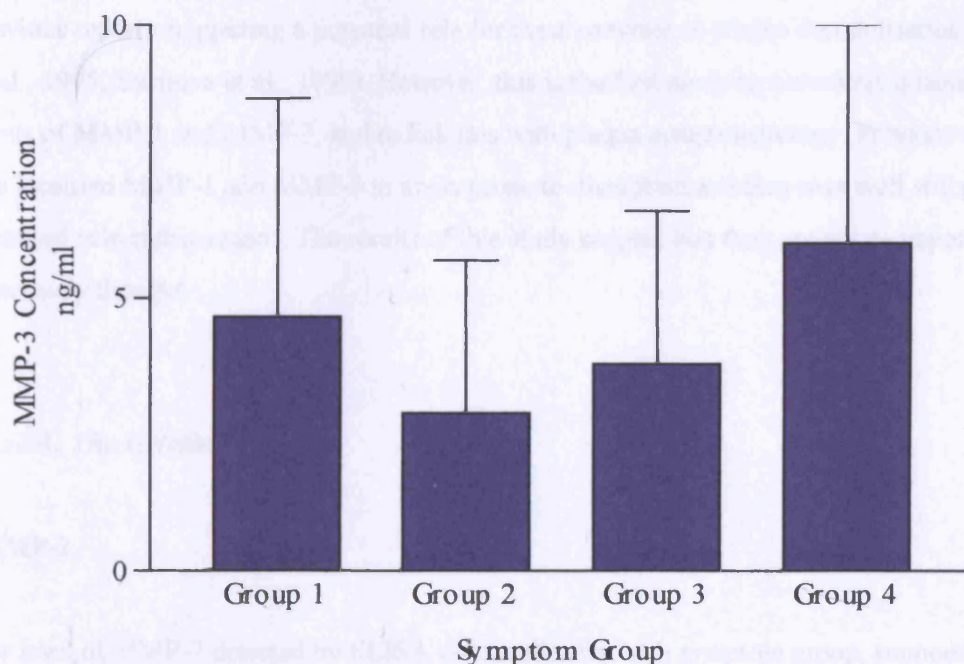


Figure 8.10: The plaque levels of MMP-1 detected by ELISA (median and upper quartile).

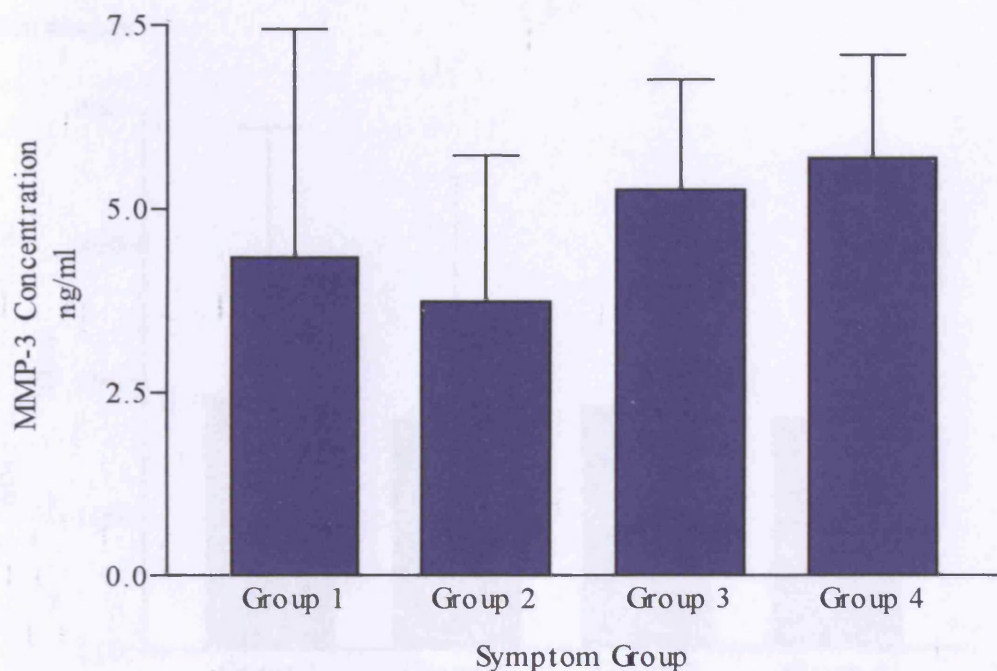


Figure 8.11: The plaque levels of MMP-3 detected by ELISA (median and upper quartile).

The results regarding the levels of MMP-1 and MMP-3 in carotid plaques seem to contradict previous reports suggesting a potential role for these enzymes in plaque destabilisation (Nikkari et al., 1995; Sukhova et al., 1999). However, this is the first study to accurately quantify the levels of MMP-1 and MMP-3, and to link this with plaque symptomatology. Previous work has localised MMP-1 and MMP-3 to areas prone to disruption and they may well still play a localised role in this regard. The results of this study suggest that they are not as important as previously thought.

ELISA: The Gelatinases

MMP-2

The level of MMP-2 detected by ELISA was similar for each symptom group, supporting the results of zymography (Figure 8.12). The majority of plaques had levels under 400 ng/ml, though in 6 plaques, levels of >1000ng/ml were detected. Unlike MMP-9, there was no

significant difference between the plaque and control tissue levels of MMP-2. This suggests that MMP-2 is constitutively expressed throughout arterial tissue with no localised increase within plaques.

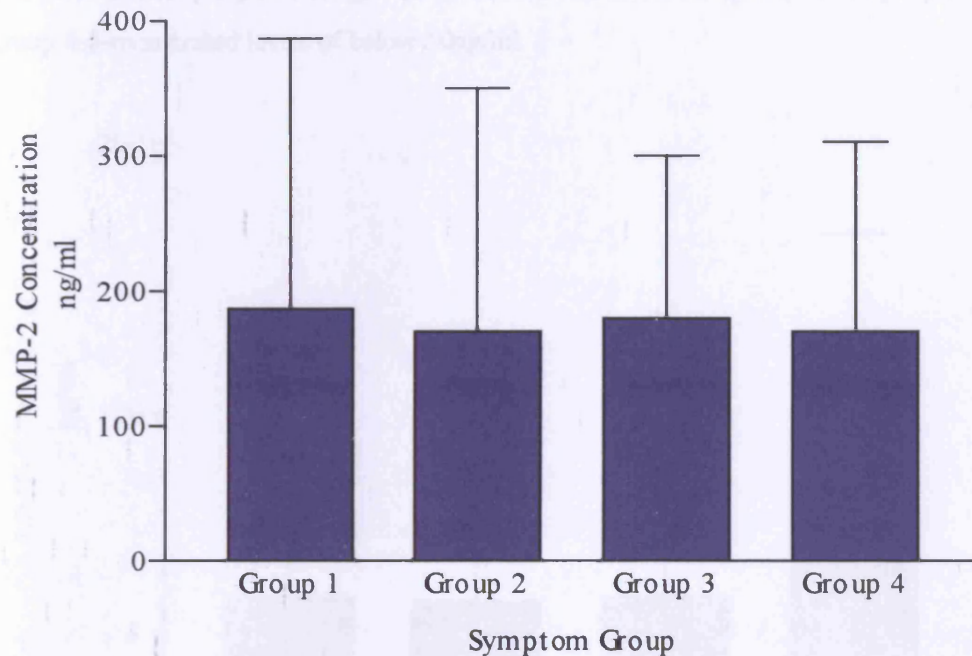


Figure 8.12: *The plaque levels of MMP-2 detected by ELISA.*

As with MMP-1 and MMP-3 the results regarding the level of MMP-2 in plaques, detected by both zymography and ELISA, seem to contradict previous studies suggesting a role for MMP-2 in plaque disruption (Li et al., 1996; Galis et al., 1994b). This is the first study to quantify MMP-2 levels by ELISA, previous studies merely demonstrating the presence of MMP-2 within plaques. It seems unlikely to be the major factor involved in plaque proteolysis.

MMP-9

Symptom Groups

MMP-9 levels fell within a smaller range than MMP-2, reaching a maximum of 290 ng/ml in symptom group 4, as illustrated in Figure 8.13. The levels in symptom group 4 were four times

higher than the other 3 symptom groups and this was highly significant ($p=0.003$, Kruskal Wallis). Again this supported the results of zymography.

The level in most plaques from groups 1, 2 and 3 fell below 50 ng/ml, but only 4 plaques from group 4 demonstrated levels of below 50ng/ml.

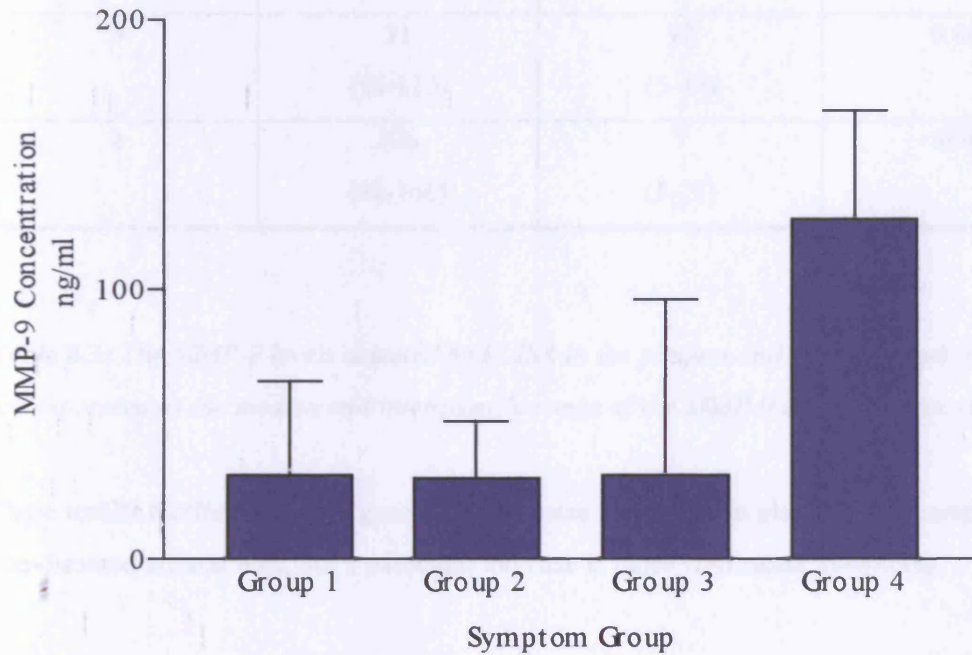


Figure 8.13: The plaque levels of MMP-9 detected by ELISA. The levels in symptom group 4 were significantly higher than the other 3 symptom groups ($p=0.003$, Kruskal Wallis).

Plaque versus Control

As demonstrated by zymography, the level of MMP-9 measured by ELISA for each group was significantly higher than the control tissue levels. The most dramatic difference was observed in group 4 with an 18-fold difference in levels as shown in Table 8.3.

Group	Plaque [MMP-9]	Control [MMP-9]	p value
1	31 (25-75)	13 (6-21)	0.003
2	30 (17-51)	5 (3-7)	0.001
3	31 (15-110)	12 (5-44)	0.001
4	126 (62-166)	7 (5-21)	<0.001

Table 8.3: *The MMP-9 levels detected by ELISA in the plaques and control tissue (results are expressed as the median and interquartile range of the MMP-9 concentration, ng/ml).*

These results therefore showed a generalised increase in MMP-9 in plaque tissue compared to non-diseased arterial wall, but a particular increase in those with recent symptoms.

Correlation Between ELISA and Zymography

The results of zymography for MMP-2 and MMP-9 were compared to the ELISA results. While the ELISA kits are proven to be highly sensitive and reproducible for a variety of tissue types, including human tissue homogenates, there were no previous publications regarding their use with atherosclerotic tissue. We therefore performed correlation and regression analysis on the 2 sets of data for MMP-2 and MMP-9.

The scatter plots are shown in Figures 8.14 and 8.15, along with lines of best fit. The results of zymography for the latent forms of MMP-2 and MMP-9 were found to correlate closely with the ELISA results. For MMP-2 and MMP-9 respectively, the Spearmans Correlation Coefficient was 0.711, $p=0.001$ ($r^2=0.673$, $p=0.001$), and 0.582, $p=0.01$ ($r^2=0.202$, $p=0.01$). This validates the use of ELISA for carotid plaque homogenates, without negating the benefit of zymography in differentiating between active and latent forms of the enzymes.

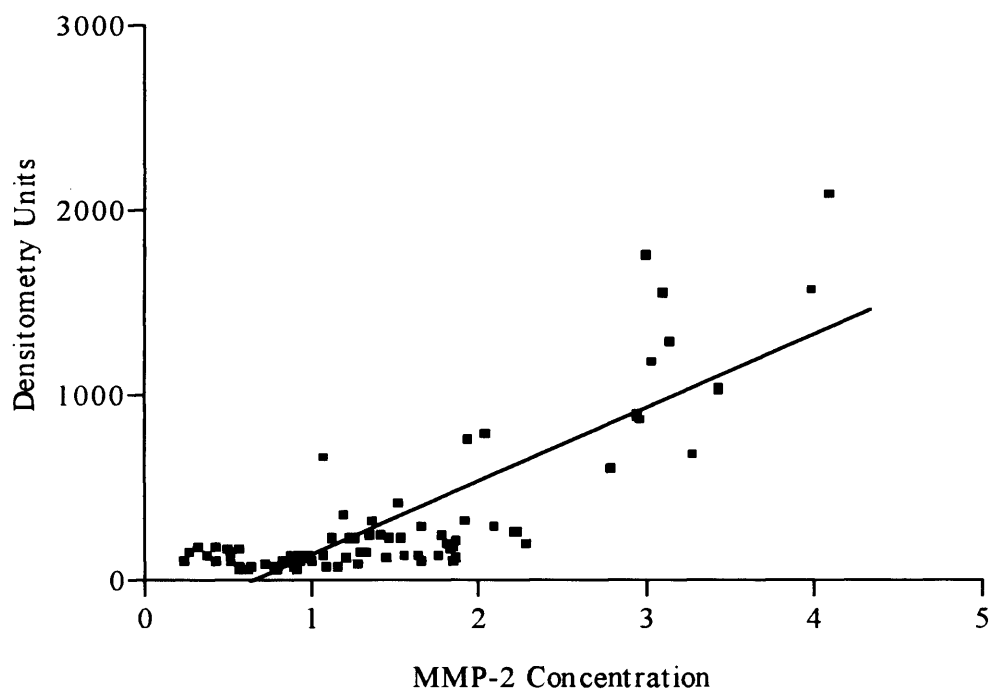


Figure 8.14: Correlation between MMP-2 as measured by ELISA (MMP-2 concentration ng/ml) and zymography (densitometry units) ($r^2=0.673$, $p=0.001$).

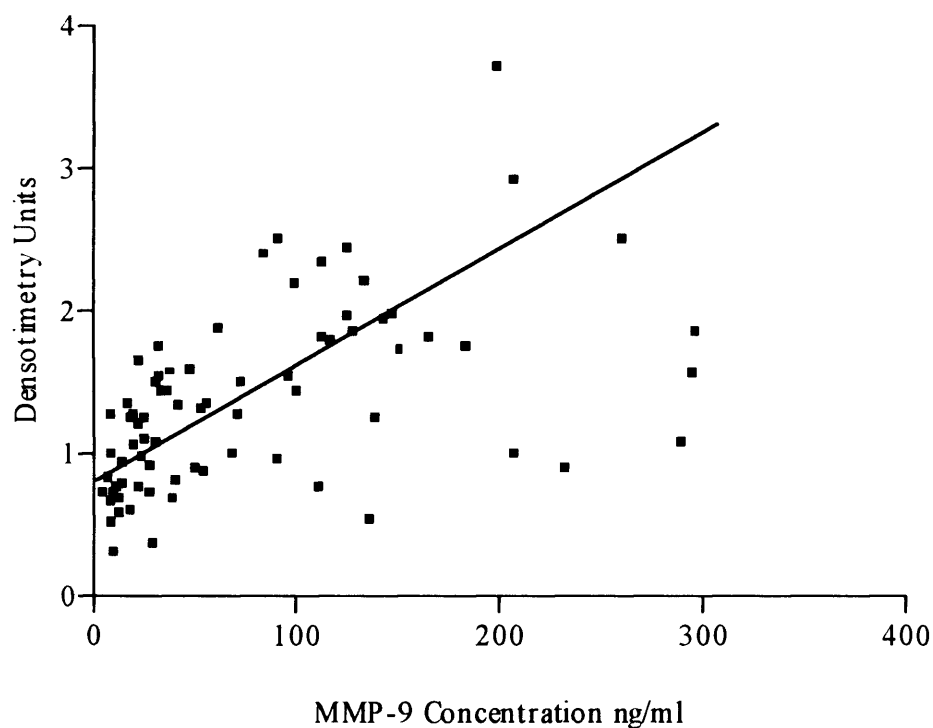


Figure 8.15: Correlation between MMP-9 as measured by ELISA (MMP-9 concentration ng/ml) and zymography (densitometry units) ($r^2=0.202$, $p=0.01$).

The TIMPs

The levels of TIMP-1 and TIMP-2 are shown in Figures 8.16 and 8.17. The levels of TIMP-1 were much higher than the levels of TIMP-2, with levels over 2000ng/ml detected in 4 plaques from groups 3 and 4, but less than 250ng/ml of TIMP-2 in the majority of the plaques. There was a trend towards increased levels of TIMP-1 in the more recent symptom groups but this failed to reach statistical significance. No difference was observed between the groups for TIMP-2.

It may well be that much of the TIMP-2 was bound to pro-MMP-2 and the TIMP-2 ELISA assay fails to detect this complex. The MMP-2 assay does detect the pro-MMP-2/TIMP-2 complex, and this would explain the relatively high levels detected using the MMP-2 assay relative to the low levels detected with the TIMP-2 assay. The TIMP-1 assay on the other hand recognises total TIMP-1 including that complexed with MMP-s.

There was a significant increase in the level of TIMP-1 in each of the symptom groups when compared to the control vascular tissue as shown in Table 8.4 (results are expressed as the median and interquartile range of the TIMP-1 concentration, ng/ml).

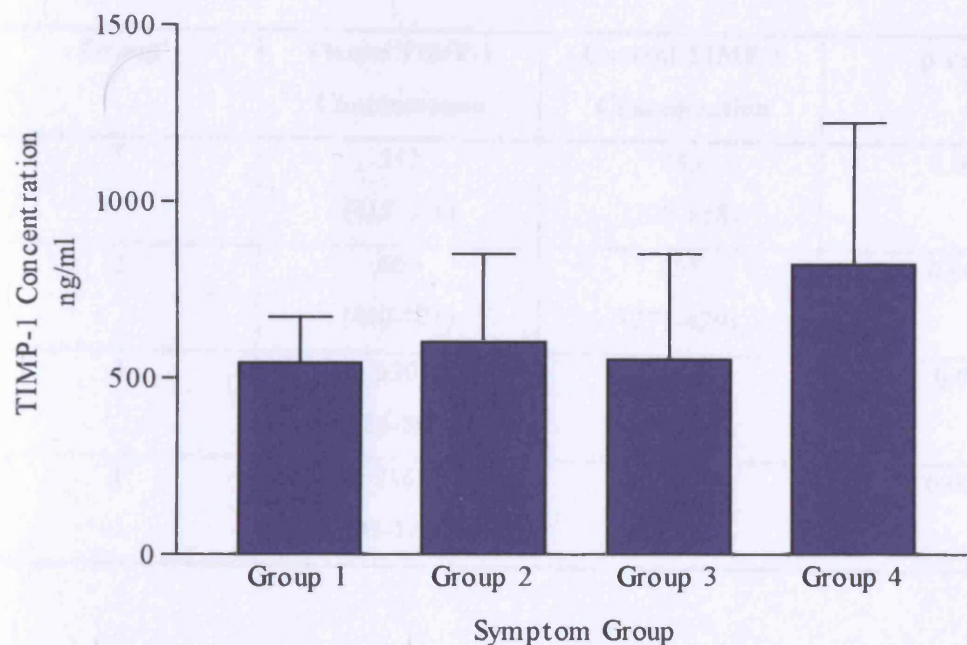


Figure 8.16: The plaque levels of TIMP-1 detected by ELISA. There were no significant differences between the groups.

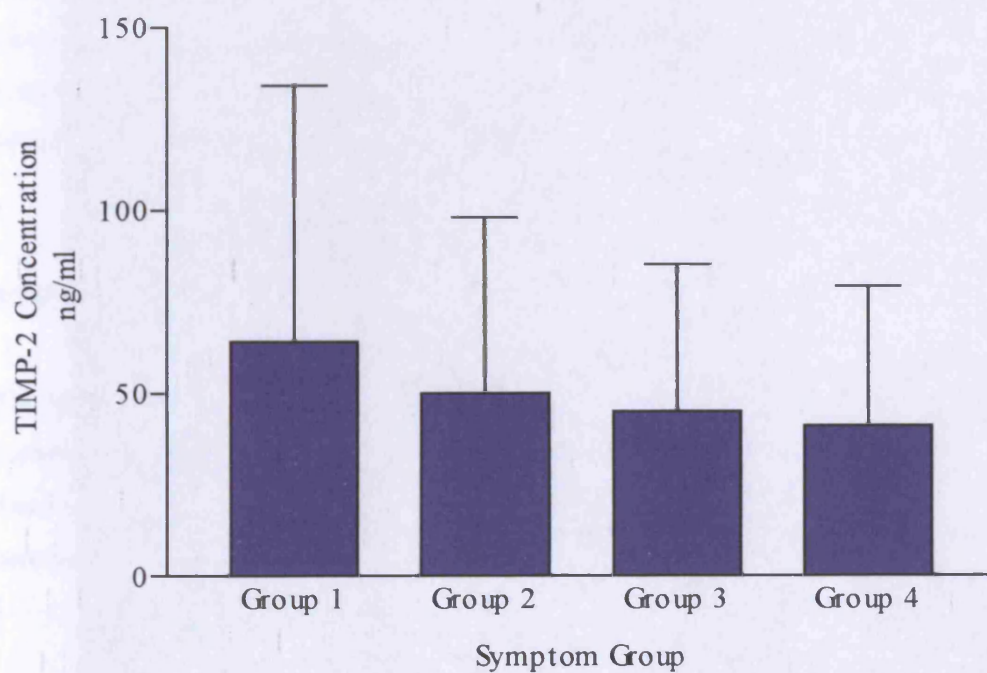


Figure 8.17: The plaque levels of TIMP-2 detected by ELISA. There were no significant differences between the groups

Group	Plaque TIMP-1 Concentration	Control TIMP-1 Concentration	p value
1	542 (418-659)	285 (228-468)	0.005
2	600 (440-871)	355 (271-429)	0.007
3	530 (335-897)	319 (230-545)	0.03
4	816 (593-1267)	288 (250-497)	0.001

Table 8.4: The TIMP-1 levels detected by ELISA in the plaques and control tissue (results are expressed as the median and interquartile range of the MMP-9 concentration, ng/ml).

This suggested a generalised increase in the expression of TIMP-1 in plaque tissue compared to non-diseased arterial wall. This may be in response to the increase in MMP-9 expression. However the fact that the TIMP-1 level was not significantly higher in Group 4 plaques compared to the other groups suggests that there may be an imbalance between the increases in MMP-9 and TIMP-1.

MMP-1/TIMP-1 Complex

The MMP-1/TIMP-1 complex assay detects activated MMP-1 that has subsequently been complexed with TIMP-1. It does not recognise free active MMP-1, TIMP-1 or pro-MMP-1.

The levels both in plaque and control tissue were very low, with no significant differences between the groups (Figure 8.18).

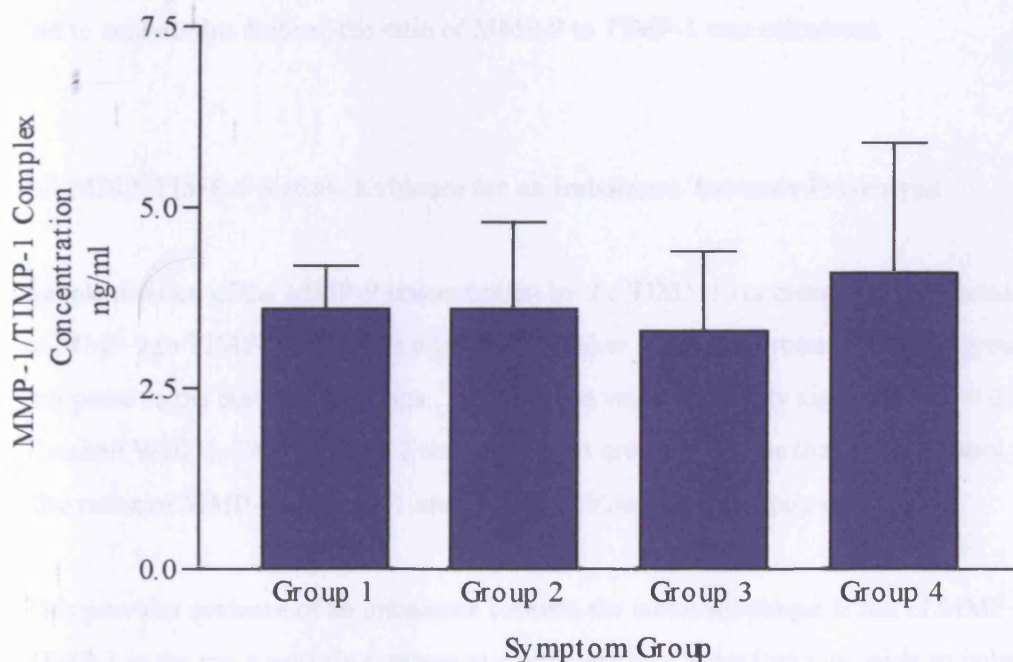


Figure 8.18: The plaque levels of MMP-1/TIMP-1 complex detected by ELISA

Summary of ELISA and Zymography Results.

The level of pro-MMP-9 detected by ELISA was four times higher in plaques from patients with recent symptoms compared to other plaques. Zymography supported these results, but also showed a significant increase in the level of active MMP-9 in these plaques. The level of MMP-9 was significantly higher in all plaque groups compared to control vascular tissue.

There were no differences between the groups in the level of MMP-1, MMP-2 or MMP-3. MMP-1 and MMP-3 were detected at very low levels, with similar amounts found in control tissue. This explains the failure to detect caseinolytic activity on zymography. While higher levels of MMP-2 were measured, again the levels were similar to the controls.

The level of TIMP-1 was higher in the most recent symptom group but this failed to reach significance. However, the increase in TIMP-1 compared to control tissue did reach significance for all symptom groups. There was no difference between the groups in the levels of TIMP-2. This suggested an imbalance between the level of MMP-9 and its major inhibitor, but to analyse this further, the ratio of MMP-9 to TIMP-1 was calculated.

8.3 MMP/TIMP-1 Ratios- Evidence for an Imbalance Towards Proteolysis

Simple division of the MMP-9 concentration by the TIMP-1 concentration calculated the ratio of MMP-9 to TIMP-1. The ratio was 3 times higher in the most recent symptom group compared to the other three groups. This increase was statistically significant ($p=0.016$, Kruskal Wallis). The ratio was 8 times higher in group 4 plaques than in the control tissue. The ratios of MMP-9 to TIMP-1 are shown in Table 8.5 and Figure 8.19.

This provides evidence of an imbalance between the increased plaque levels of MMP-9 and TIMP-1 in the more recently symptomatic plaques. This is the first time such an imbalance has been described. Whilst MMP-9 has been detected in vulnerable areas of the plaque (Brown et al., 1995), the level has not been quantified and associated to symptomatology previously. By comparing it to the TIMP-1 level, the major physiological inhibitor of MMP-9, this provides evidence of a shift in the equilibrium within the plaque towards increased proteolysis which would predispose to plaque disruption.

Group	Plaque [MMP-9]	Plaque [TIMP-1]	Ratio
1	31 (25-75)	542 (418-659)	0.064 (0.051-0.130)
2	30 (17-51)	600 (440-871)	0.058 (0.036-0.086)
3	31 (15-110)	530 (335-897)	0.051 (0.041-0.103)
4	126 (62-166)	816 (593-1267)	0.142 (0.064-0.178)

Table 8.5: *The ratio of MMP-9 to TIMP-1.*

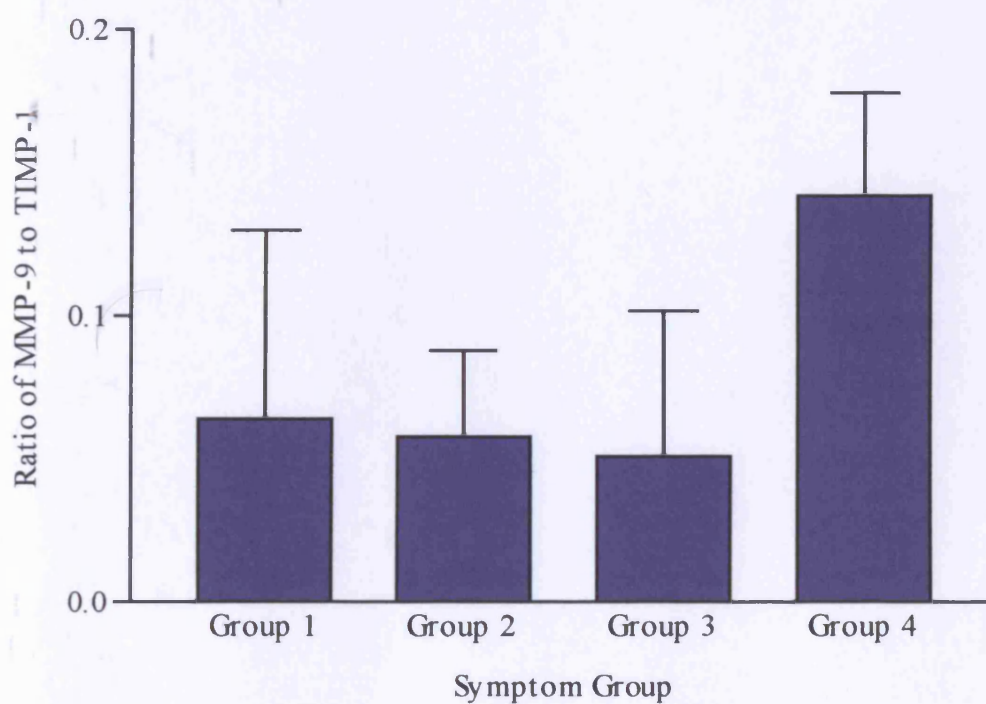


Figure 8.19: *The ratio of MMP-9 to TIMP-1.*

8.4 Summary

The level of latent and active MMP-9 was raised in plaques from patients with recent symptoms in the absence of raised TIMP-1 levels. This was reflected in a significant increase in the ratio of MMP-9 to TIMP-1, suggesting an imbalance towards increased proteolysis within these plaques. This would predispose a plaque to increased risk of disruption and may represent a target for pharmacotherapy aimed at plaque stabilisation.

Further evidence for plaque instability was sought by histological analysis and emboli detection. The results are outlined in the following chapter.

CHAPTER NINE

RESULTS

MMP Levels in Association With Other Features of Plaque Instability

9.1 MMP-9 Levels and Spontaneous Embolisation

9.2 Histological Features of Instability and MMP-9 Levels

Plaque Rupture

Intraplaque Haemorrhage

Plaque Necrosis

Fibrous Plaques

Summary of Histology

9.3 Blood Levels of MMPs

9.4 Summary

9.1 MMP-9 Levels and Spontaneous Embolisation

The level of MMP-9 was significantly higher in those plaques from patients in whom spontaneous particulate embolisation was detected than those without (median 71.7 ng/ml versus 31.5 ng/ml, $p=0.017$, Kruskal Wallis). This is demonstrated graphically in Figure 9.1. Although there was an increase in active MMP-9 detected on zymography in the embolising group this just failed to reach statistical significance. There were no differences in the other MMP subtypes nor TIMP levels in relation to embolisation.

The ratio of MMP-9 to TIMP-1 was significantly higher in the embolising group (median ratio 0.1 versus 0.06, $p=0.01$), again suggesting an imbalance between the increased level of MMP-9 and its inhibitor.

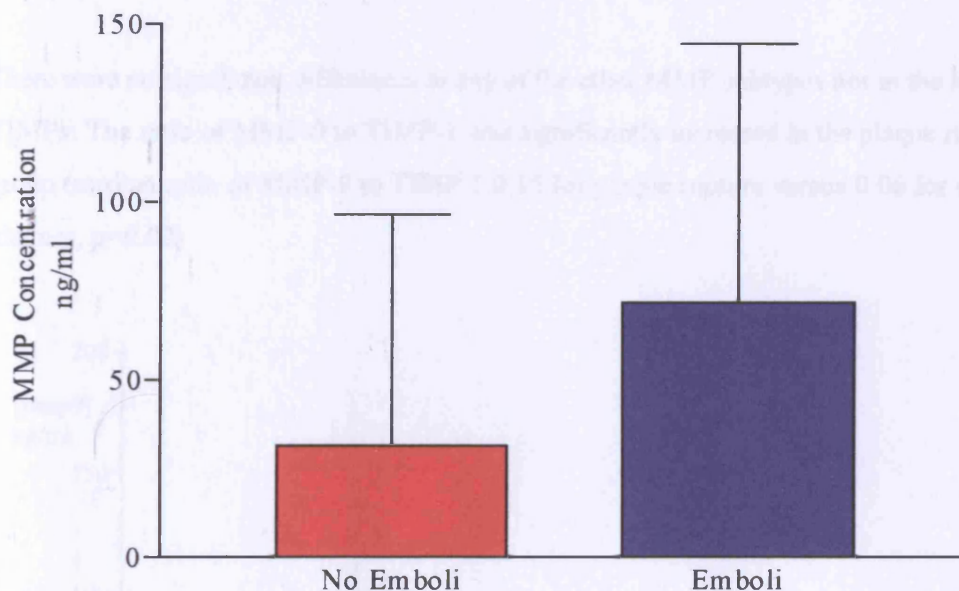


Figure 9.1: The level of MMP-9 in embolising plaques ($n=21$) compared to non-embolising plaques ($n=49$)(median and upper quartiles).

As discussed previously, embolisation is arguably the most sensitive marker of plaque instability, correlating with neurological damage in the form of deteriorating cognitive function(Gaunt et al., 1996b). The clear increase in plaque MMP-9 in embolising plaques is therefore particularly important.

9.2 Histological Features of Instability and MMP-9 Levels

Three histological features of plaque instability were associated with significant increases in MMP-9 levels, namely plaque rupture, intraplaque haemorrhage and plaque necrosis. These are illustrated graphically in Figures 9.2-9.4.

Plaque Rupture

For plaques with histological evidence of rupture, the level of pro-MMP-9 detected on ELISA was threefold that measured in plaques with an intact cap (median 91.1ng/ml versus 33.9ng/ml respectively, $p=0.03$). Furthermore, zymography revealed a significant increase in the level of active MMP-9 in plaques with cap rupture (median zymogram ratio 1.51 units versus 0.96 units, $p=0.02$).

There were no significant differences in any of the other MMP subtypes nor in the level of TIMPs. The ratio of MMP-9 to TIMP-1 was significantly increased in the plaque rupture group (median ratio of MMP-9 to TIMP-1 0.15 for plaque rupture versus 0.06 for intact plaques, $p=0.02$).

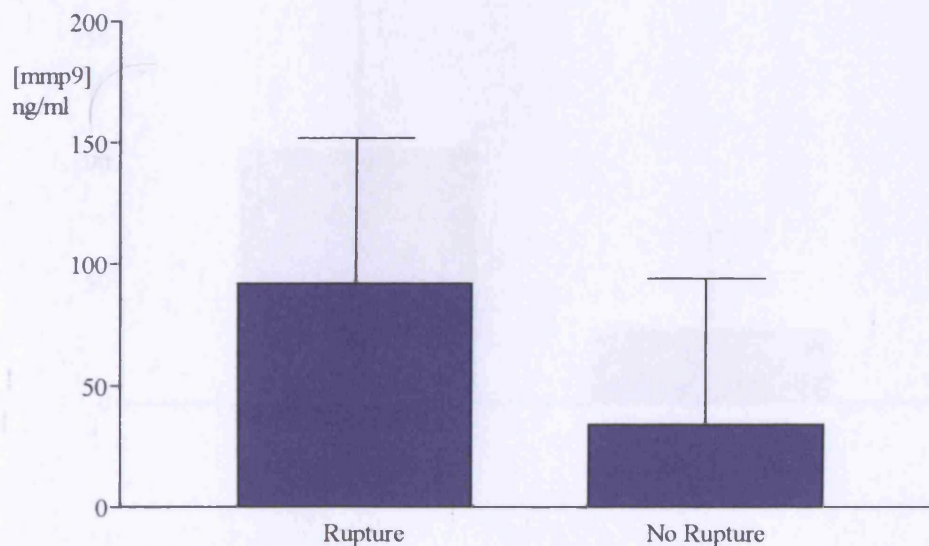


Figure 9.2: The level of MMP-9 in plaques with histological evidence of plaque rupture was significantly higher than those plaques with an intact cap ($p=0.03$).

Intraplaque Haemorrhage

For plaques with histological evidence of intraplaque haemorrhage, the level of pro-MMP-9 detected on ELISA was more than threefold that measured in plaques without haemorrhage (median 103.6 ng/ml versus 30.3 ng/ml respectively, $p=0.009$). As seen with plaque rupture, zymography revealed a significant increase in the level of active MMP-9 (median zymogram ratio 1.46 units versus 0.98 units, $p=0.02$).

There were no significant differences in any of the other MMP subtypes nor in the level of TIMPs, and the ratio of MMP-9 to TIMP-1 was significantly increased in the haemorrhage group (median ratio of MMP-9 to TIMP-1 0.11 for intraplaque haemorrhage versus 0.03 for plaques without, $p=0.007$).

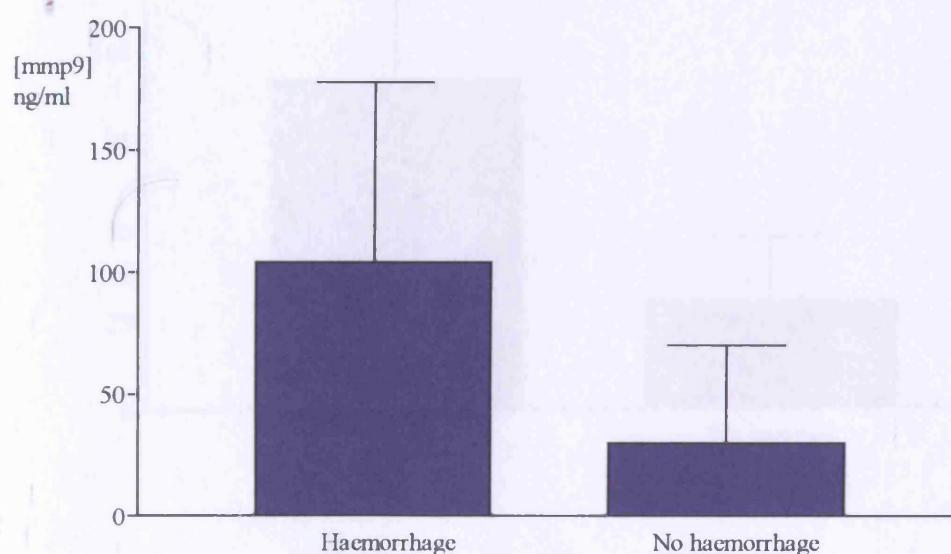


Figure 9.3: The level of MMP-9 in plaques with histological evidence of intraplaque haemorrhage was significantly higher than those plaques without ($p=0.009$).

Plaque Necrosis

Plaques with histological evidence of necrosis also demonstrated a threefold increase in pro-MMP-9 detected on ELISA (median 91.0 ng/ml versus 30.3 ng/ml respectively, $p=0.003$), and again zymography revealed a significant increase in the level of active MMP-9 (median zymogram ratio 1.36 units versus 0.94 units, $p=0.05$).

There was no significant difference in any of the other MMP subtypes nor in the level of TIMPs, and the ratio of MMP-9 to TIMP-1 was significantly increased in the plaques with necrosis group (median ratio of MMP-9 to TIMP-1 0.10 for plaque necrosis versus 0.05 for plaques without, $p=0.03$).

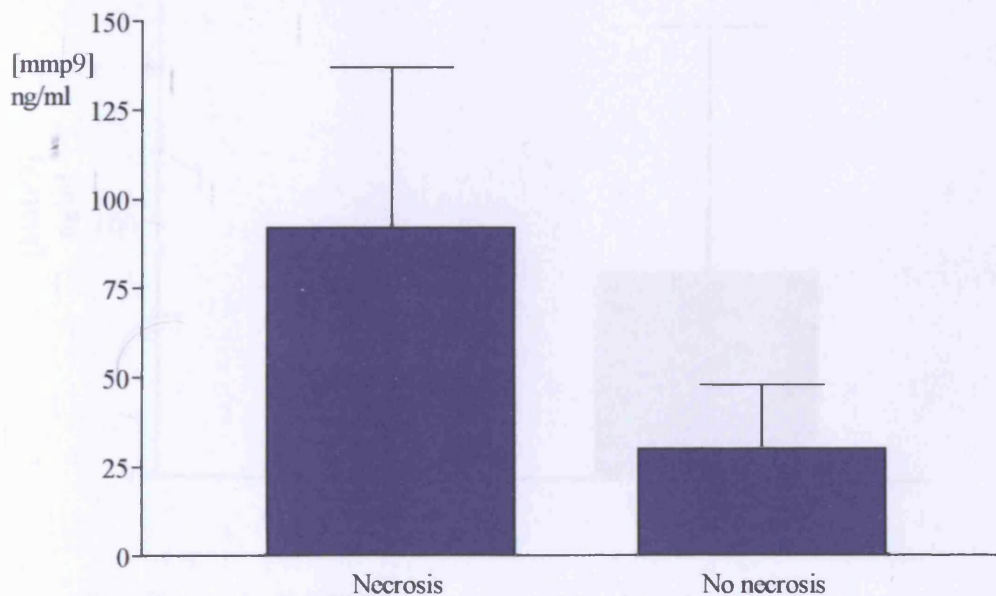


Figure 9.4: The level of MMP-9 in plaques with histological evidence of necrosis was significantly higher than those plaques without ($p=0.003$).

Fibrous Plaques

Predominantly fibrous plaques demonstrated a lower level of MMP-9 detected by ELISA than other plaques (median 30.8 ng/ml versus 81.4 ng/ml respectively, $p=0.01$; Figure 9.5).

Zymography failed to show any difference in active MMP-9, nor was there a difference in the other subtypes or TIMP levels.

The ratio of MMP-9 to TIMP-1 was significantly lower in the fibrous plaques compared to other plaques (0.05 versus 0.11, $p=0.049$).

No other histological features were associated with differences in MMP or TIMP levels. Nor did histological features of the plaque influence control vascular tissue MMP levels.

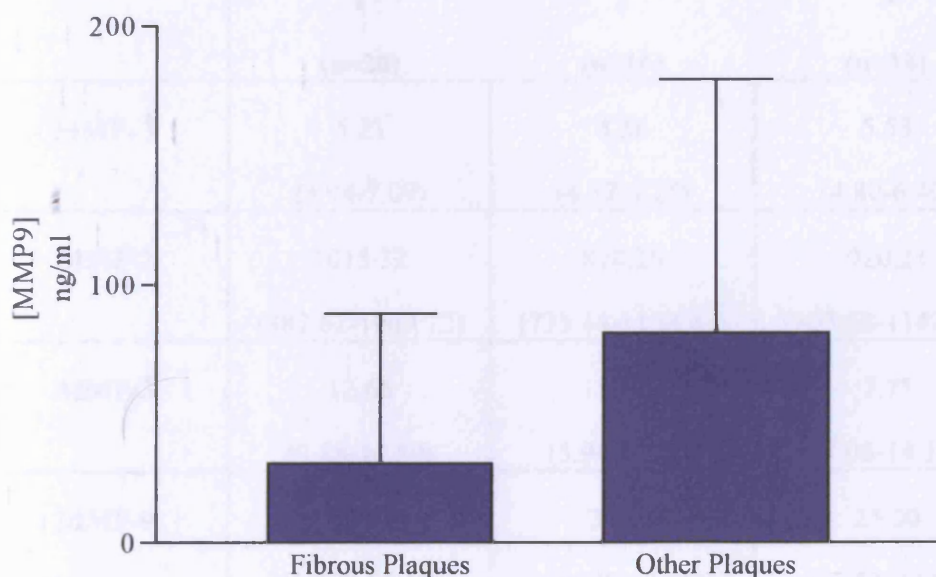


Figure 9.5: *The level of MMP-9 in fibrous was significantly lower than other plaques ($p=0.01$).*

Summary of Histology

The histological features now accepted as evidence of acute disruption, and therefore identifying the most unstable plaques, were detected in similar proportions to previous large histopathology studies (Carr et al., 1996). The level of MMP-9 was significantly higher in plaques with these features, namely plaque rupture, necrosis and intraplaque haemorrhage. Fibrous plaques deemed to be 'more stable' had a significantly lower level of MMP-9. This is

the first time a quantitative association has been made between histological evidence of instability and a proteolytic enzyme capable of inducing such instability.

9.3 Blood Levels of MMPs and TIMPs

The plasma levels of MMPs and TIMPs were measured by ELISA and are shown in Table 9.1.

There were generally low levels of MMP-1, MMP-1/TIMP-1 complex and MMP-3 detected, with higher levels of the gelatinases, in particular MMP-2. There were also relatively high levels of TIMP-1 and TIMP-2 detected. There were no differences between the symptom groups in the levels of any of the subtypes including MMP-9, nor in the ratio between MMP and TIMP-1.

	Symptom Group			
	1 (n=20)	2 (n=16)	3 (n=18)	4 (n=21)
MMP- 1	5.21 (3.94-7.09)	4.56 (4.37-5.25)	5.53 (4.80-6.40)	5.57 (3.52-7.88)
MMP-2	1015.32 (887.62-1069.72)	870.26 (735.14-1153.41)	920.21 (805.48-1142.55)	742.59 (599.59-1083.37)
MMP-3	12.66 (9.88-16.89)	8.64 (5.94-10.36)	7.75 (7.08-14.33)	9.69 (7.19-13.81)
MMP-9	32.59 (14.77-53.42)	35.70 (26.20-42.60)	25.90 (17.53-37.89)	31.79 (22.13-51.58)
TIMP-1	702.86 (468.95-871.15)	866.20 (537.19-937.38)	717.06 (568.86-1038.13)	702.91 (626.90-1317.21)
TIMP-2	516.80 (474.99-600.11)	475.48 (461.63-564.86)	532.47 (479.30-577.74)	506.03 (454.69-554.93)
MMP-1/TIMP-1 Complex	3.36 (1.28-5.10)	0.39 (0-0.76)	2.88 (1.11-3.34)	2.70 (0.67-4.81)

Table 9.1: *The plasma concentration of MMP/TIMP subtypes determined by ELISA.*

There were no significant differences between the groups.

None of the histological features of plaque instability had a significant effect upon the circulating level of MMPs. However, the plasma MMP-9 level was significantly higher in those patients in whom spontaneous embolisation was detected on TCD monitoring. In those patients with emboli detected the median serum MMP-9 concentration was almost double that for patients without emboli (43.6 ng/ml versus 26.2 ng/ml respectively, $p=0.03$), as shown in Figure 9.6. Analysis of variance revealed that the plasma MMP concentration was the only significant predictor of embolisation ($p=0.03$).

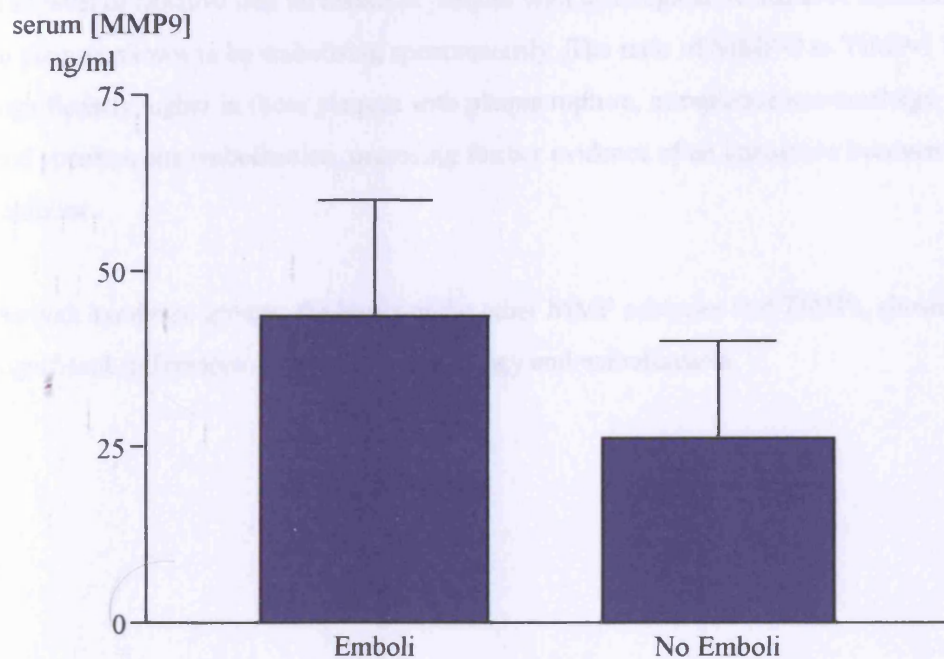


Figure 9.6: *The plasma level of MMP-9 was significantly higher in those patients with spontaneous embolisation on TCD monitoring than those without ($p=0.03$).*

This may represent one of two phenomena. Firstly it may reflect a systemic manifestation of the localised increase in plaque MMP-9 levels, occurring in tandem with intense localised inflammation and tissue degradation. There may be an increased release of the enzyme into the circulation from the plaque itself, or by circulating macrophages involved in the inflammatory response promoting plaque degradation. In acute coronary syndromes for example, monocytes in the systemic circulation undergo activation and may represent a source of enzyme. (Jude et al., 1994)

The increase may on the other hand represent a marker of end organ ischaemia in the form of thrombo-embolic cerebral damage. Experimental models of focal cerebral ischaemia have shown an association between the degree of cerebral injury and cerebral MMP levels(Mun-Bryce and Rosenberg, 1998)

9.4 Summary

The level of MMP-9 was increased in plaques with histological evidence of instability, and also in plaques shown to be embolising spontaneously. The ratio of MMP-9 to TIMP-1 was also significantly higher in those plaques with plaque rupture, intraplaque haemorrhage, necrosis and spontaneous embolisation, providing further evidence of an imbalance between enzyme and inhibitor.

As with symptom groups, the levels of the other MMP subtypes and TIMPs, showed no significant differences with regard to histology and embolisation.

CHAPTER TEN

RESULTS

Localisation of Production and Expression of MMP-9 to the Plaque

10.1 Immunostaining for MMP-9

10.2 In Situ Hybridisation for MMP-9 Messenger RNA

10.3 Reverse Transcription Polymerase Chain Reaction

10.4 Summary

10.1 Immunostaining for MMP-9

Immunostaining confirmed the presence of MMP-9 within the plaques and demonstrated intense localised areas of staining in areas known to be vulnerable to acute disruption, particularly the cap and shoulder of the plaque (Figure 10.1). This corresponded to areas exhibiting intense inflammatory cell infiltrates.

Those plaques in which low levels of the enzyme were measured demonstrated very weak staining for the enzyme (Figure 10.2). Further quantification was deemed unnecessary in view of the zymography and ELISA results. The immunohistology could have been quantified by point counting or computerised image analysis. This is semi-quantitative at best and would not have added any further useful information.

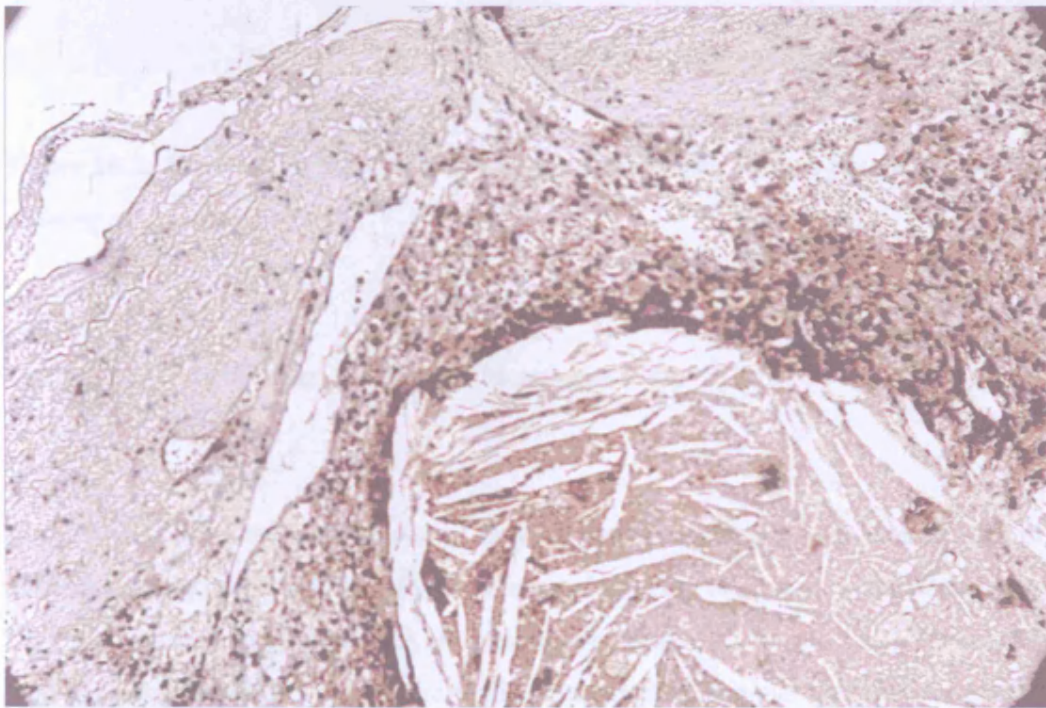


Figure 10.1: *Immunostain for MMP-9 in a plaque from a highly symptomatic patient. This demonstrates intense staining in the shoulder and cap of the plaque, surrounding the lipid rich necrotic core (x50 magnification).*

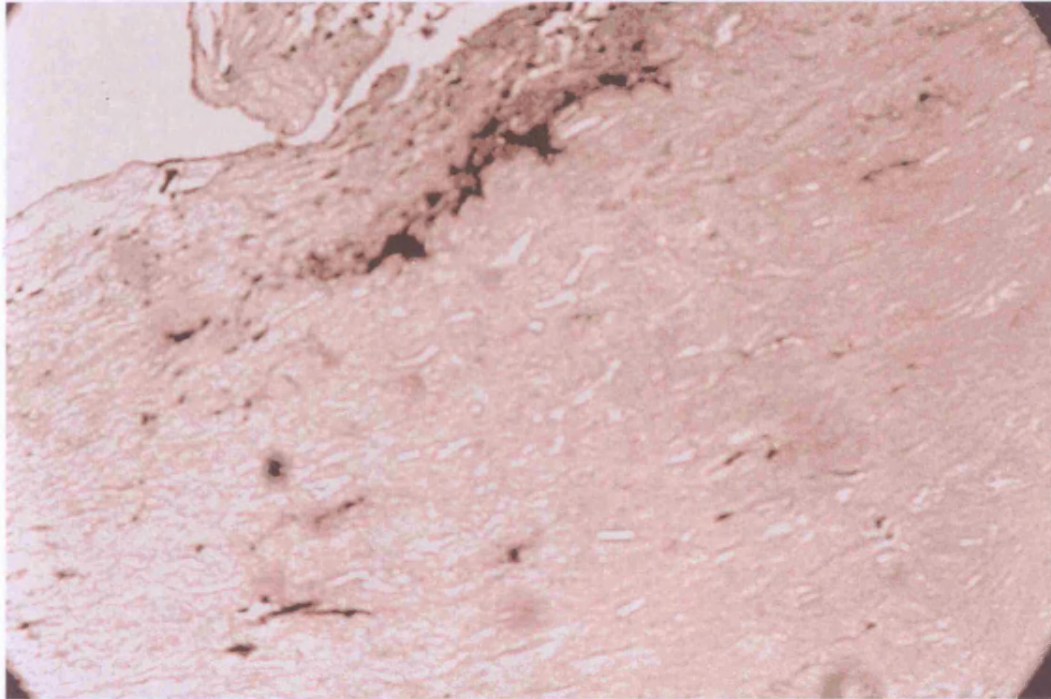


Figure 10.2: *Immunostaining for MMP-9 in an asymptomatic fibrous plaque with minimal staining (x50 magnification).*

10.2 In Situ Hybridisation for MMP-9 Messenger RNA

In situ hybridisation exhibited an almost identical pattern of staining, with intense areas of positive staining for MMP-9 mRNA in the cap and shoulder of plaques which demonstrated high levels of the enzyme on ELISA and zymography (Figures 10.3 and 10.4). Those plaques with low levels of MMP-9 on immunostaining demonstrated minimal positivity for MMP-9 mRNA (Figure 10.5).

For similar reasons to those discussed with regard to immunostaining, the in situ hybridisation was not quantified any further.

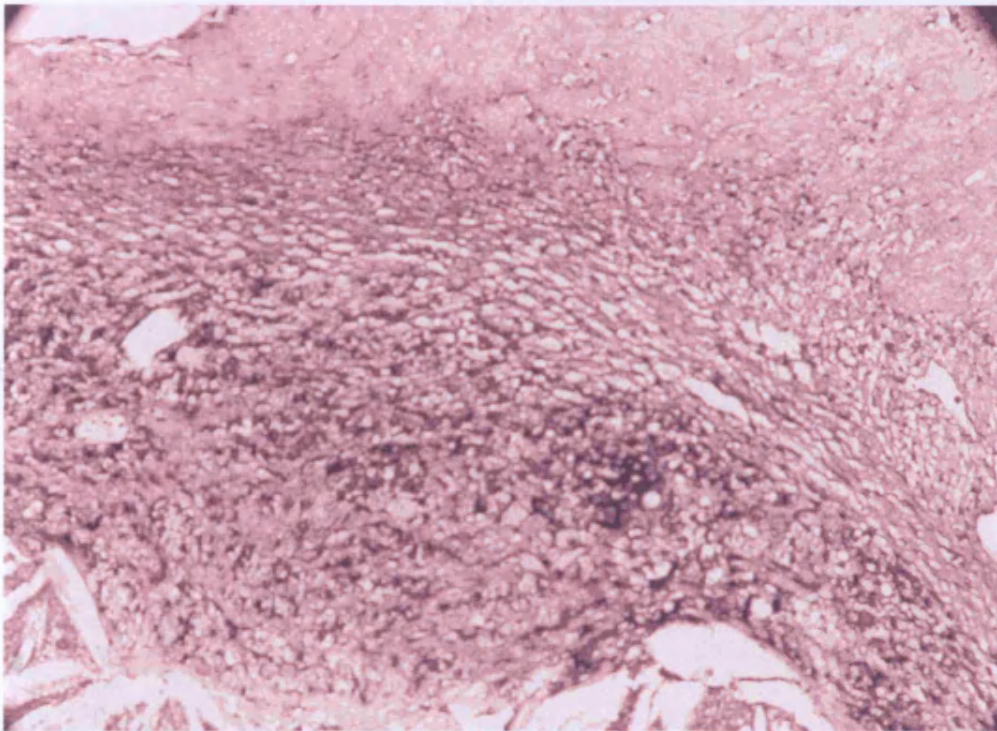


Figure 10.3: *In situ hybridisation for MMP-9 mRNA. This section from a highly symptomatic plaque demonstrates intense positive staining in the shoulder of the plaque, an area of intense inflammatory cell infiltration that is vulnerable to disruption (x50 magnification).*

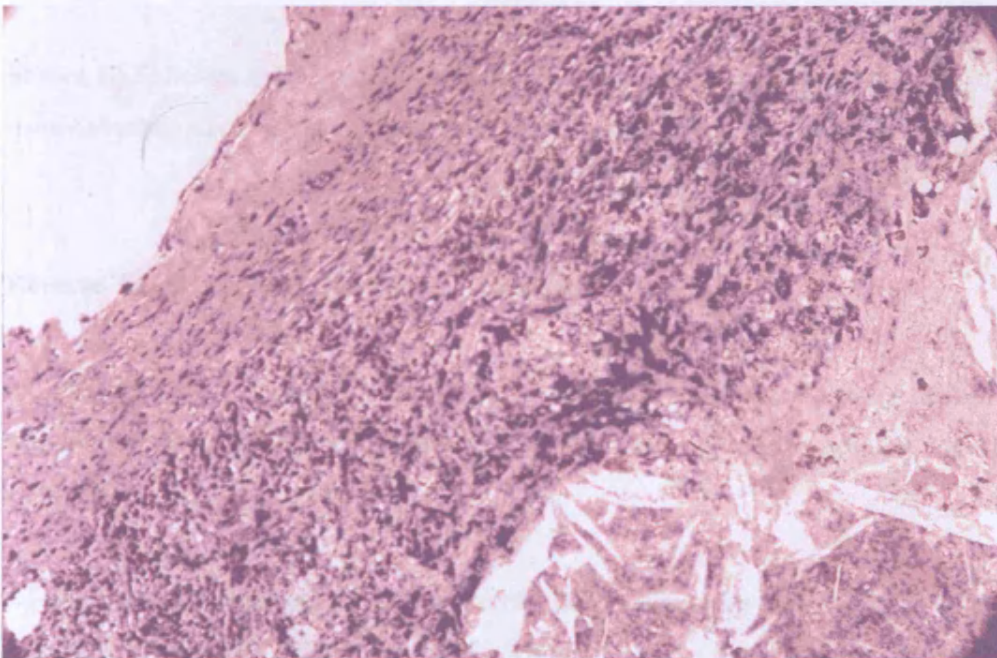


Figure 10.4: *Further in situ hybridisation for MMP-9 mRNA from a highly symptomatic plaque, showing an area of intense staining in the cap of the plaque (x50 magnification).*

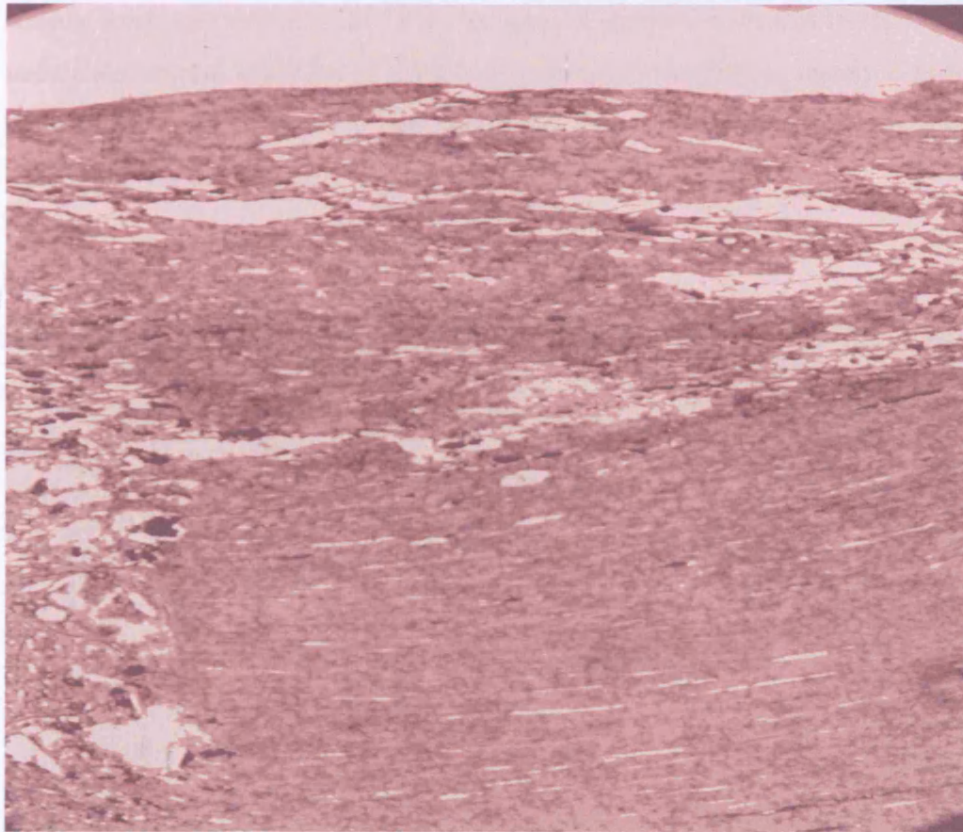


Figure 10.5: *In situ* hybridisation for MMP-9 mRNA in an asymptomatic fibrous plaque, demonstrating minimal positive staining (x50 magnification).

Reverse Transcription Polymerase Chain Reaction

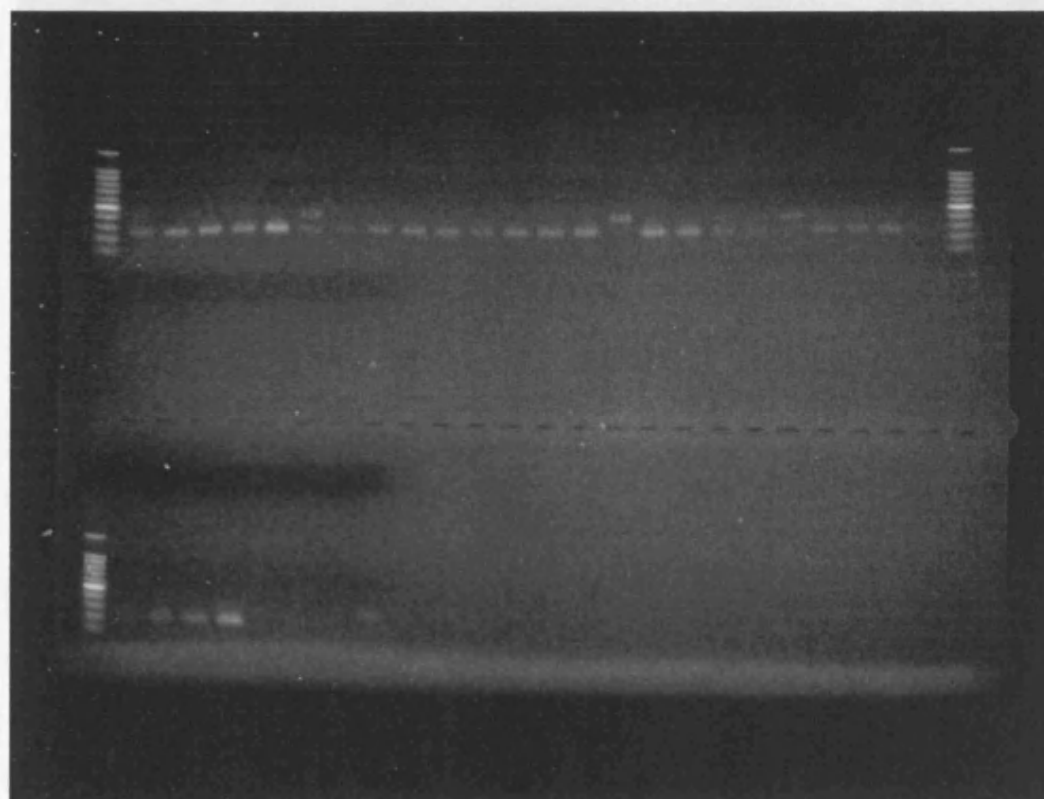
RT-PCR for MMP-9 mRNA confirmed the results of the *in situ* hybridisation, demonstrating expression within the majority of plaques of varying intensity, as shown by the white bands on a black background in Figure 10.6.

RT-PCR also demonstrated the expression of TIMP-1 within the plaques, again with varying intensity (Figure 10.7). This technique of mRNA detection is qualitative and so the degree of expression was not quantified further.

More quantitative mRNA techniques were not employed for a number of reasons. Firstly very limited amounts of plaque tissue were available for RNA analysis, with the priority given to

enzyme quantification and histological analysis. Secondly, the aim of the study was to quantify enzyme levels and activity and to then, on demonstrating the increased levels of MMP-9 and an imbalance between MMP-9 and TIMP-1 levels in unstable plaques, merely to provide evidence that MMP-9 and TIMP-1 were expressed within the plaque. Detailed quantification of expression was deemed unnecessary.

(a)



(b)

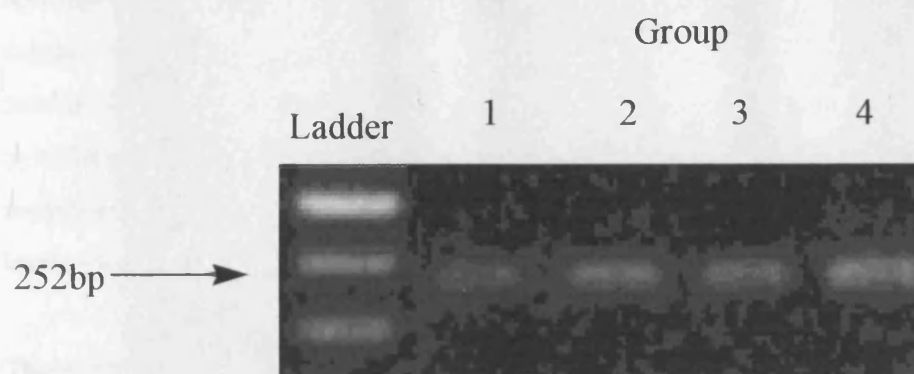


Figure 10.6: *PCR amplification demonstrating MMP-9 expression (a) and higher magnification of representative plaques from each symptom group (b).*

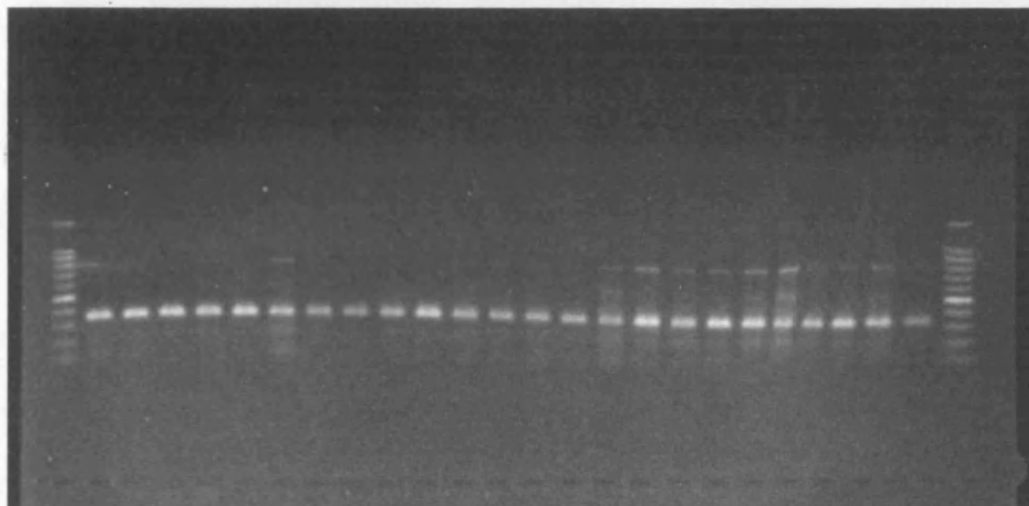


Figure 10.7: *PCR amplification demonstrating TIMP-1 expression in carotid plaques.*

Summary of Results

The combination of zymography and ELISA demonstrated an increased level of latent and active forms of MMP-9 in the most unstable carotid plaques based on patient symptomatology, spontaneous cerebral embolisation and histological feature of instability such as plaque rupture, intraplaque haemorrhage and plaque necrosis. There was no parallel increase in the level of TIMP-1, and the ratio of MMP-9 to TIMP-1 was higher in unstable plaques. In situ hybridisation and RT-PCR confirmed that the enzyme, along with TIMP-1, was produced locally within the plaque in areas most vulnerable to acute disruption. However, serum MMP-9 levels were raised in patients who were undergoing spontaneous embolisation.

These results strongly support a role for MMP-9 in the acute de-stabilisation of the plaque leading to the onset of clinical ischaemic events.

SECTION FOUR

DISCUSSION AND FUTURE WORK

CHAPTER ELEVEN

DISCUSSION

The atherosclerotic plaque is a dynamic structure, undergoing continuous remodelling of the extracellular matrix upon which its structural integrity depends. Acute changes within the plaque, such as intra-plaque haemorrhage, cap rupture and cap ulceration, are preludes to the onset of clinical ischaemic events (Falk et al., 1995). Much evidence suggests that each phase of the atherosclerotic process is mediated by matrix metalloproteinases (Dollery et al., 1995), the main physiological regulators of the extracellular matrix.

Integrally involved in all aspects of tissue remodelling, the MMPs are secreted in a latent pro-enzyme form by a range of cell types, including inflammatory cells, fibroblasts and smooth muscle cells. They subsequently require activation by limited proteolysis, a process that is tightly controlled by specific TIMPs. As well as their activity in normal tissue remodelling, interest has focused on a role in the resorption of periodontal structures in periodontal disease (Page, 1991), the destruction of joints in rheumatoid arthritis (Harris, 1990) and the local invasiveness of malignancies (Parsons et al., 1997). MMP genes are abundantly expressed in these disease states and, more recently, evidence has developed in a role for MMPs in vascular diseases.

The enzymes are absolutely dependent upon zinc for their activity, and are inhibited by metal chelating agents. Furthermore, their role in pathological states has led to the development of specific MMP inhibitors (Denis and Verweij, 1997).

Previous studies have documented a relationship between the sites of coronary plaque rupture and macrophage infiltration (Moreno et al., 1994). Macrophages are potent producers of MMPs, in particular MMP-9. Further studies have identified MMP-9 in human coronary atherosclerotic lesions obtained at atherectomy (Brown et al., 1995), and have suggested an association between active enzyme synthesis and unstable angina. Increased expression of MMP-1 and MMP-3 has been reported within carotid plaques in association with macrophage and mast cell infiltration (Nikkari et al., 1995, Henney et al., 1991) whereas active synthesis of MMP-2 has been observed within aortic plaques (Li et al., 1996). Galis et al suggested that a localised increase in expression of MMP-9 in the most vulnerable regions of atherosclerotic plaques may promote destabilisation (Galis et al., 1995). No previous study has accurately

quantified the levels of the major subtypes within human plaques.

In this study, we hypothesised that a localised imbalance in the level of MMPs and their inhibitors may be associated with plaque instability and the onset of clinical events. The aim of the study was to establish the level of the major MMP/TIMP- subtypes within carotid plaques and correlate them with clinical and histological features of plaque instability.

The present study has clearly demonstrated a localised increase in the concentration of MMP-9 in the most unstable carotid plaques based on recent focal cerebral ischaemic events, and has demonstrated expression of MMP-9 within this tissue. Plaques with other features highly indicative of instability, namely histological evidence of plaque rupture and intraplaque haemorrhage, and the detection of spontaneous cerebral particulate embolisation, also demonstrated a significant increase in the level of MMP-9 when compared to plaques from less symptomatic patients. Despite this elevation of MMP-9, the level of TIMP-1 was not increased, causing a local imbalance between the enzyme and its major physiological inhibitor. The change in the ratio of enzyme to inhibitor proved to be statistically significant for patient symptomatology, spontaneous embolisation and the main histological features of plaque instability.

Similar results have been demonstrated in aneurysms, where despite an increase in the level of both MMP-9 and TIMP-1, the ratio between the 2 increases significantly compared to non-aneurysmal tissue (Tamarina et al., 1997). Similar results have been described for MMP-2 and TIMP-2 (Knox et al., 1997).

Plaque enzyme levels, in those patients with symptoms between 4 weeks and 6 months prior to endarterectomy, were similar to those detected in asymptomatic plaques. This strongly suggests that MMP-9 plays a key role in acute plaque disruption leading to the onset of symptoms. Recent work in patients with acute coronary syndromes supports this theory, showing a sharp but transient increase in plasma levels of MMP-9 (Kai et al., 1998). We found no difference in plasma MMP levels relating to recent symptoms, though this may well be related to the broader cohort of patients within each group.

Our data demonstrated that there was no significant difference between the plaque groups in the levels of the other major MMP- subtypes, in particular MMP-1, MMP-2 (both active and latent), and MMP-3. Previous studies have identified the presence of several MMPs within

atherosclerotic plaques with authors suggesting a role in plaque destabilisation. Henney et al demonstrated the presence of mRNA for MMP-3 in coronary plaques by in situ hybridisation (Henney et al., 1991) whilst the expression of MMP-1 in carotid plaques was described by Nikkari (Nikkari et al., 1995). Galis and colleagues described localised increases in MMP-9 surrounding the lipid core of plaques, particularly in the shoulder and cap of the plaque, and demonstrated the presence of both latent and active forms of the enzyme by zymography (Galis et al., 1995). However, in all of these studies, the enzyme levels were not quantified, though the latter reported an increase in overall proteolytic activity in the vulnerable regions of the plaque by in situ zymography. Additionally, in previous studies, the procurement of specimens was not standardised, and the MMP activity could not be related to patient symptomatology or other features of instability.

In this study, the levels of both MMP-1 and MMP-3 were very low and similar to the levels detected in control tissue. Previous studies have localised these enzymes to areas prone to plaque disruption and they may well be involved in the cascade of enzyme activity, despite their low levels. Further work is required to establish the precise levels of individual enzyme activity in these areas.

As discussed briefly in the results section of this thesis, much of the MMP-2 within the plaque may be bound to TIMP-2, accounting for the high levels of MMP-2 detected by ELISA and low levels of TIMP-2.

Brown et al demonstrated the presence of MMP-9 in coronary plaques retrieved at atherectomy, with a similar level of positive immunostaining in those from patients with stable and unstable angina (Brown et al., 1995). However, they documented intracellular localisation of the enzyme in all patients with unstable angina, indicating active synthesis, compared to only 30% of those with stable angina. Again, however, they failed to quantify enzyme levels or determine the production of TIMPs.

In contradistinction to the previous study by Brown, which did not identify MMP-9 in normal internal mammary artery by immunostaining, the current study has demonstrated that MMP-9 was present in the normal common carotid control tissue. The concentration in all plaques was significantly higher than in the corresponding control tissue. The level of TIMP-1 was higher in the plaques when compared to the control tissue, reaching significance in all but the asymptomatic group. There have been conflicting reports of TIMP-1 levels in aortic

atherosclerotic disease, with some showing a modest increase in the level of TIMP-1 and others reporting no change (Galis et al., 1995; Thompson et al 1995). The current study has shown that, in all but the most recently symptomatic group, there was an increase in both MMP-9 and TIMP-1, perhaps maintaining a physiologic balance, with just the most recently symptomatic plaques demonstrating an imbalance between MMP-9 and TIMP-1 levels. The precise cause of this localised imbalance is unclear.

The site of plaque rupture has been characterised by an intense inflammatory infiltration, consisting predominantly of macrophages, foam cells and T-lymphocytes and it seems certain that this inflammatory infiltration plays a key role in the destabilisation of the plaque (Buja and Willerson, 1994). It appears that this infiltrate within the plaque undergoes a period of activation at the time of acute coronary syndromes, and the associated release of proteolytic enzymes may lead to destabilisation of the plaque (Van der Wal et al., 1998).

Genetic factors may determine which individuals are prone to develop acute complications of atherosclerosis. Previous work has identified a genetic variation in the MMP-3 promoter associated with the progression of coronary atherosclerosis (Ye et al., 1995) and it is possible that such genetic variation affects other members of the MMP family. More recently, a polymorphism has been detected in the MMP-9 promoter gene and a possible association with severity of atherosclerosis (Zhang et al., 1999). This, in the light of the results of this study, may be particularly relevant in predicting plaque development and requires further investigation.

There may also be a role for other factors in the cascade of MMP regulation, in particular the plasminogen system (Allaire et al., 1998), oxidised low density lipoproteins (Stemme and Hanson, 1994) and Chlamydia pneumoniae (Muhlestein et al., 1998), all undergoing further investigation.

Plasmin represents the major physiological activator of MMPs, but despite work in tumour invasion suggesting a link with MMPs, the role of plasmin in atherosclerosis is unclear. It is known that macrophages express uPA and its receptor and inhibition in vitro prevents matrix degradation (Estreicher et al., 1989).

Oxidised LDL has a strong link with the development of atherosclerosis and may play a role in acute disruption. The recent finding that macrophages in culture exposed to oxidised LDL up-

regulate the production of MMP-9, but down regulate expression of TIMP-1, is particularly interesting (Xu et al., 1999). It may be that this event in vitro precipitates acute disruption but such an association may be very difficult to prove.

The link between infectious agents and atherosclerosis remains the topic of much debate. Results from serological studies present contrasting data, but of the agents studied, Chlamydia remains the organism of most interest. It is of note that Chlamydial heat shock protein 60 regulates TNF α and MMP expression in cultured macrophages (Kol et al., 1999).

Other proteolytic enzymes, in particular other members of the MMP family, are likely to be involved in the cascade process within the plaque. Those that warrant further investigation include MMP-8, MMP-12 and MMP-13. MMP-12 has been described in both plaques and aneurysms but not quantified (Halpert et al., 1996; Curci et al., 1999). It is the most potent metallo-elastase described to date, produced predominantly by macrophages, and therefore may be important in plaque destabilisation.

Interest has also focussed recently on the role of cyclooxygenase in plaque disruption. The production of MMPs and other proteolytic enzymes by activated macrophages occurs through a prostaglandin (PG) E₂-cAMP dependent pathway involving the modulation of cyclooxygenase (Corcoran et al., 1994). Two isoforms of this enzyme have been identified, COX-1 and COX-2. They convert arachidonic acid to prostaglandin H₂, the precursor of other prostaglandins and thromboxanes. The levels of prostaglandins and thromboxanes are markedly enhanced in mature plaques (Reilly et al., 1999). COX-1 is a constitutively expressed enzyme responsible for maintaining low levels of prostaglandins that are thought to be involved in housekeeping functions such as vascular homeostasis. In contrast, COX-2 is induced in response to cell activators such as pro-inflammatory cytokines and growth factors. It has been detected in atheromatous plaques, localised to macrophages in the vulnerable regions such as the shoulder and cap (Schonbeck et al., 1999). Induction of COX-2 production in macrophages leading to the production of PGE₂ is involved in the signal transduction pathway leading to MMP production by the same cells.

Aspirin is an inhibitor of both COX-1 and COX-2 and has been used for years to reduce the risk from cardiovascular disease. The benefit has been linked to its effect upon platelet function, the reduction in thromboxane production preventing platelet aggregation. However, its anti-inflammatory properties may also be important (Speir et al., 1998).

Understanding the relationship between this pathway and its regulatory effect on the induction of MMPs and other proteolytic enzymes may be crucial to the formulation of pharmacotherapy strategies aimed at plaque stabilisation. Recently developed COX-2 inhibitors may have the potential to disrupt this relationship and reverse the cycle of events leading to plaque disruption. COX-2 inhibition limits the expansion of experimental aortic aneurysms, a disease characterised by inflammation and macrophage COX-2 and MMP expression (Miralles et al., 1999). Lipoproteins, a key stimulant of the plaque inflammatory process, have been shown to stimulate macrophage prostaglandin production through COX-2 raising the further possibility that agents targeting lipid metabolism, in particular statins, may also inhibit this pathway (Vinals et al., 1997).

Justification for the patient grouping was derived from the results of transcranial detection of cerebral emboli and plaque histology. A number of studies have highlighted the importance of spontaneous cerebral embolisation in determining the most unstable plaques (Gaunt et al., 1996), and in this study more than 50% of the recently symptomatic patients had evidence of such emboli. This may well represent the most sensitive marker of plaque instability as it detects those plaques that are in the process of undergoing disruption and thrombosis. The demonstration of a significant rise in the level of serum MMP-9 in patients with spontaneous embolisation is therefore of particular interest.

This may represent one of two phenomena. Firstly it may reflect a systemic manifestation of the localised increase in MMP-9 levels. There may be an increased release of the enzyme into the circulation from the plaque itself, or by circulating macrophages involved in the inflammatory response promoting plaque degradation. In acute coronary syndromes for example, monocytes in the systemic circulation undergo activation and may represent a source of enzyme (Jude et al., 1994).

The increase may on the other hand represent a marker of end organ ischaemia in the form of thrombo-embolic cerebral damage. CT scans were not performed in this study to look specifically for evidence of infarction because previous studies of particulate embolisation have found that CT is unhelpful in this regard (Gaunt et al., 1996a). A more sensitive measure of end organ damage may be psychometric testing and previous studies have shown a clear link between particulate embolisation and a deterioration in psychological scoring (Gaunt et al., 1996b).

Experimental models of focal cerebral ischaemia have shown an association between the degree of cerebral injury and cerebral MMP levels(Mun-Bryce and Rosenberg, 1998). Middle cerebral artery occlusion in rats has been shown to cause an increased MMP-2 and MMP-9 activity(Rosenberg et al., 1996), whilst further studies have shown that MMP-9 may contribute to post-infarction oedema and haemorrhage(Rosenberg et al., 1990). More recently, MMP-2 and MMP-9 levels have been demonstrated to increase very early within ischaemic basal ganglia of non-human primates in parallel with evidence of neurone injury(Ji et al., 1999). In humans the level of cerebral MMP-9 has been shown to be high in association with an inflammatory infiltration predominantly of leukocytes in patients who died shortly after a stroke(Anthony et al., 1997).

Increased cerebral levels of MMP-9 may therefore contribute to early disruption of the microvascular basal lamina and accentuate neuronal injury. The cellular source of cerebral MMPs remains unclear but focal cerebral ischaemia may influence plasma MMP levels and vice versa.

Ji Hóe Heo and colleagues demonstrated a transient rise in circulating MMP-9 levels in a rat model of middle cerebral artery occlusion(Ji et al., 1999). However, there are no previous reports linking plasma MMP levels with markers of plaque instability. Circulating levels of both MMP-2 and MMP-9 have been shown to increase in patients with acute coronary syndromes. Kai et al measured plasma MMP levels in 33 patients with acute coronary syndromes, 17 patients with stable angina and 17 normal controls(Kai et al., 1998). Early increases in circulating levels of both MMP-2 and MMP-9 were demonstrated in patients with acute coronary syndromes, higher than both normal controls and those with stable angina.

Work in aneurysms has shown an association between serum levels of MMP-2 and cerebral aneurysms (Todor et al., 1998), but more interestingly serum levels of MMP-9 are increased in patients with abdominal aortic aneurysms and the level is particularly high in those patients with multiple aneurysms (McMillan et al., 1999). It is unclear from these studies whether the aneurysms represent the source of the circulating enzymes and this clearly demands further investigation.

Previous work has suggested an association between systemic markers of inflammation and atherosclerosis, including C-reactive protein, but they failed to provide discriminatory

diagnostic power over and above existing risk factors. Serum MMP-9 levels in this study provided an independent indicator of increased risk of embolisation and may represent a useful marker of plaque instability.

Further justification for the patient grouping was evident regarding plaque histology, with the incidence of plaque rupture and haemorrhage higher in plaques from Group 4. However, both features were identified in patients from each of the symptom groups. Previous studies have identified coronary plaque disruption at post-mortem in patients who died of non-cardiac causes (Davies 1992), and thus it seems probable that such acute changes can occur in the carotid vessels without causing symptoms. Conversely, histological features of instability were not detected in some patients in the most recently symptomatic group. One limitation of the study was that, by necessity, only a small proportion of each plaque was examined microscopically and it may well be that features were missed in some patients. This may particularly apply to the identification of cap foam cells, shown in previous studies to be more common in symptomatic plaques, which may be missed by examining a small number of individual sections.

Despite these reservations, the results of this study are otherwise similar to most published series relating to histological features of instability, such as that by Carr and colleagues (Carr et al., 1996). The level of MMP-9 is significantly higher in those plaques with such features of instability, in particular plaque rupture or fissuring, intraplaque haemorrhage and plaque necrosis. The ratio of MMP-9 to TIMP-1 was significantly higher also, demonstrating an imbalance between enzyme levels and inhibition. The level of MMP-9 was significantly lower in those plaques described as predominantly fibrous and therefore inherently more stable.

Whilst many of the factors which predispose to the early development of the atherosclerotic lesion have been identified, there remains uncertainty as to the reasons why, after years of indolent growth, a plaque should suddenly undergo the acute changes which predispose to the onset of symptoms. This study has confirmed the presence of MMP-9 in both active and latent forms within carotid plaques retrieved at carotid endarterectomy. The concentration of this enzyme was significantly higher in the most unstable carotid plaques, as determined by patient symptomatology, spontaneous particulate cerebral embolisation and histological features of instability.

Immunostaining and *in situ* hybridisation revealed intense synthesis of MMP-9 in those areas

of the plaque most at risk of acute disruption, namely the cap and shoulder of the plaque, in turn associated with inflammatory infiltration. Furthermore the level of MMP-9 within the plaque was raised in all patient groups when compared to paired control tissue, as were the concentrations of its major inhibitor, TIMP-1. Similar increments in the levels of both MMP-9 and TIMP-1 were observed in all groups except for highly symptomatic patients, the plaques of which exhibited an increased MMP-9 / TIMP-1 ratio. Such an imbalance has the potential to cause the acute plaque disruption which precedes the onset of symptoms in both the coronary and cerebral circulations.

The current treatment options for both advanced carotid and coronary disease remain surgical and radiological, and despite improvements in techniques associated with intense policies of quality control, they still have associated mortality and morbidity. Particularly with carotid disease, patients who may not develop further ischaemic complications may be treated unnecessarily because it is impossible to determine which patients are most likely to benefit from treatment.

MMP-9 therefore represents an attractive target for pharmacotherapy to prevent plaque destabilisation and a variety of pharmaceutical agents have been shown to inhibit MMP activity. It is recognised that a causal relationship could only be concluded from a formal randomised controlled trial of an MMP inhibitor in patients with significant stenoses.

CHAPTER TWELVE

FUTURE WORK

The findings of this study suggest that for the first time, it may be possible to minimise the risk of acute plaque disruption and thereby reduce the risk of thrombo-embolic stroke. There is now sufficient evidence to link MMP activity and plaque destabilisation but it remains to be seen whether MMP inhibition alters the natural history of the plaque. If this were the case, it could have immense clinical implications in the long term prevention of stroke and myocardial infarction. Before this hypothesis can be tested in a multi-centre randomised trial of patients with mild and moderate carotid disease, it is essential that we first demonstrate that MMP inhibitors can modify MMP activity within the carotid plaque. Therefore, the next stage of this work is to perform a randomised controlled trial of an MMP inhibitor in patients with significant carotid stenoses

Doxycycline is a particularly attractive agent in this regard. It has a proven long term safety in the treatment of acne vulgaris and pelvic inflammatory disease, with very few side effects or drug interactions. It has been shown that doxycycline significantly reduced MMP activity in a model of aneurysm disease with an associated reduction in the degradation of elastin (Boyle et al., 1998). Doxycycline in culture also resulted in a significant reduction in MMP9 activity in a model of vein graft stenosis with associated reduction of smooth muscle cell proliferation and neointimal thickness (Loftus et al., 1998). In both of these studies, a significant effect was seen at therapeutic levels of the drug, equivalent in vivo to a single daily dose of 100mg. Furthermore, initial studies in vitro using tissue from carotid plaques has shown a significant reduction in MMP activity and effective uptake of the agent into plaque tissue.

There are a number of initial questions that need to be answered:

- 1) Whether doxycycline reduces plaque MMP activity compared to a placebo.
- 2) Whether doxycycline therapy causes plaque modification by comparing histology and preoperative embolisation rate between those receiving doxycycline and placebo.
- 3) Whether adequate tissue penetration occurs in vivo at the normal therapeutic dose.

A prospective randomised double-blinded trial of once daily doxycycline versus a placebo for 8 weeks prior to carotid endarterectomy has been commenced. In total, 150 consecutive patients

undergoing carotid endarterectomy will be entered into the trial except those who do not give written consent, patients with a known carcinoma (since this may effect MMP activity) and those admitted for emergency carotid endarterectomy. The majority are patients with a symptomatic ICA stenosis of >70%, while a minority have severe asymptomatic disease. All patients are provided with a full information sheet detailing possible side effects and are required to sign a specific consent form. Patients admitted to the trial are randomised to treatment with doxycycline 100mg once daily or a placebo. Unlabelled tablets are supplied in bottles marked only by a number with randomisation by the hospital pharmacy on allocation. The medical staff and patient are blinded to the allocation.

Carotid plaques are obtained from all patients at the time of surgery and divided into 3 longitudinal portions for histological examination, quantification of MMP and doxycycline levels, and RNA extraction and quantification of MMP expression. Serum is also be obtained to quantify serum MMP and doxycycline levels.

If doxycycline is shown to penetrate the plaque and affect MMP activity, a longer term trial may be warranted in patients with mild disease to assess the longer term effects of MMP inhibition on plaque development.

Other pharmacological agents are also worthy of study in models of atherosclerosis. Evidence points towards a link between oxidised LDL and MMP activity through macrophage mediated upregulation of expression, and this raises questions about the precise mode of action of HMG-CoA reductase inhibitors. Initially thought to have a direct beneficial effect of lipid lowering on reducing the risk of stroke and myocardial infarction, the relationship now appears more complex. Fluvastatin, for example, inhibits smooth muscle cell migration and proliferation independent of the lipid lowering effect, and prevents intimal hyperplasia in a rat model of arterial injury (Corsini et al., 1995; Soma et al., 1995). The effect upon MMP activity, and also perhaps COX-2 activity, requires investigation. Specific MMP inhibitors may also be useful but are currently limited by the potential for side effects.

Regarding COX-2 and other factors involved in the inflammatory reaction within unstable plaques, a detailed study is due to commence to investigate the relationship with MMP activity. Plaques will be collected from a consecutive cohort of 100 patients undergoing carotid endarterectomy. Plaque and systemic levels of COX-2, MMPs and pro-inflammatory cytokines will be measured and the following end points assessed:

- 1) The concentration and expression of COX-2 within plaques.
- 2) The association between COX-2 and MMP levels and expression within plaques.
- 3) The association between COX-2 and plaque instability including symptomatology, histological features and spontaneous embolisation.
- 4) The association between MMP and COX-2 expression and the release of pro-inflammatory cytokines.
- 5) The effect in vitro upon this relationship of COX-2 inhibition and HMG-CoA reductase inhibitors.

Further work is required to establish the role of other members of the MMP family, in particular MMP-12, but also MMP-8, MMP-13 and MT1-MMP. Little is known about the level of expression of TIMP-3 and TIMP-4 within plaques and their ratio to the major MMP subtypes. There is likely to be an integrated action of a number of enzymes within the cascade of activation that leads to excessive matrix degradation. Other proteolytic enzymes also demand investigation, such as the serine proteases and cathepsins. The relationship between MMP activity and other factors within the plaque also requires clarification. The complex interactions between inflammatory and other cells within the plaque through cytokines, cellular adhesion molecules and other such substances will directly influence MMP and TIMP expression.

The association between MMP activity and other histological changes within the plaque also warrant further investigation. While angiogenesis may predispose a plaque to haemorrhage and rupture, the link with MMP activity and plaque instability remains unclear. Neovascularisation may result in increased inflammatory cell infiltration and MMP production, but the converse may also be true. A similar argument could be established for the role of cellular apoptosis that is an important feature of the mature and unstable plaque.

The rise in serum MMP-9 requires further investigation, in particular to establish the cellular source of the circulating enzyme. This could be performed by a prospective study of patients who are admitted to a stroke unit, with serial MMP assays performed in tandem with CT scans to assess the degree of cerebral injury.

The demonstration of a close association between MMP-9 and plaque instability has raised the possibility of targeting pharmacotherapy towards preventing acute plaque change. This has the

exciting potential for, in theory, reducing the risk of stroke and myocardial infarction in patients with atherosclerosis.

SECTION FIVE: APPENDICES AND REFERENCES

Appendix 1: Reagents

Homogenising Buffer

Tissue homogenising buffer was prepared by dissolving the following in sterile water:

Urea (Sigma, Poole, UK)	2mol/l
Tris HCL (Sigma, Poole, UK)	50mmol/l
NaCl (Fisons, Loughborough, UK)	1g/l
EDTA (Fisons, Loughborough, UK)	1g/l
Brij 35 (Sigma, Poole, UK)	1ml/l
Phenylmethanesulphonyl fluoride (PMSF, Sigma, Poole, UK)	0.1mmol/l
NaOH	to final pH of 7.6

Dialysis Buffer

The dialysis buffer was derived from the following dissolved in sterile water:

Tris HCL	25mmol/l
CaCl ₂ (Fisons, Loughborough, UK)	10mmol/l
BRIJ 35	0.1%
PMSF	0.1mmol/l
NaOH	to final pH of 8.5

5x Electrode Running Buffer (pH 8.3)

This solution was prepared by dissolving 15g Tris-base, 72g chromatography grade glycine and 5.0g SDS in double distilled water with gentle stirring and adjusting final volume to 1 litre. This solution was stored at 4°C. Before use the solution was warmed to 37°C if precipitation had occurred, and then diluted five-fold with double distilled water.

Incubation Buffer

The buffer was prepared by dissolving in 800 ml double distilled water the following:

Tris base	60.6g
CaCl ₂ ·2H ₂ O	14.7g
NaCl	29.2g
BRIJ	5 ml

The final volume was then made up to 1 litre with double distilled water and adjusting to pH 7.6 with 1 mol HCL. The buffer was stored at 4°C for 3 days.

10% Sodium Dodecyl Sulphate Solution

This was prepared by dissolving 10g Sodium Dodecyl Sulphate (Fisons, Loughborough, UK) in water with gentle stirring and adjusting the final volume to 100ml with double distilled water. Once prepared this solution was stored at room temperature.

10% Ammonium Persulphate Solution

This was prepared by dissolving 10g ammonium persulphate (Sigma, Poole, UK) in water with gentle stirring and adjusting the final volume to 100ml with double distilled water. Once prepared this solution was stored at room temperature.

Gel Resolving Buffer

A 1.5M Tris-HCL buffer was prepared by dissolving 27.23g Tris base in 80ml double distilled water to pH 8.8 with 1 mol HCL. The final volume was then made up to 150ml with double distilled water and the solution stored at 4°C.

Gel stacking buffer

A 0.5M Tris-HCL buffer was prepared by dissolving 6.0g Tris base in 60ml double distilled water to pH 6.8 with 1 mol HCL. The final volume was then made up to 100ml with double distilled water and the solution stored at 4°C.

10% Polyacrylamide Separating Gel

The polyacrylamide separating gel was prepared by sequentially mixing the following:

Double distilled water	1.0ml
Resolving buffer (pH 8.8)	2.5 ml
30% Acrylamide/Bis (National Diagnostics, Georgia, USA)	3.34 ml
10% SDS solution	100µg
10% ammonium persulphate	50µg
Tetramethylethylenediamine (TEMED, Sigma, Poole, UK)	5µg

Gelatin or casein substrate was incorporated when needed to a final concentration of 1 mg/ml. Casein gels need 2.5x volume of ammonium persulphate.

10% Polyacrylamide Stacking Gel

The stacking gel was prepared by sequentially mixing together the following reagents:

Double distilled water	6.1 ml
Stacking buffer (pH 6.8)	2.5 ml
30% Acrylamide/Bis solution	1.3 ml
10% SDS solution	100µg
10% ammonium persulphate	50µg
TEMED	10µg

2x Non-Reducing Buffer

8.0ml of non-reducing sample buffer was prepared by mixing together the following reagents:

Double distilled water	2.8ml
0.5M Tris-HCL	1.0 ml, pH 6.8
Glycerol (Sigma, Poole, UK)	0.8ml
10% SDS solution	3.2ml
0.2% bromophenol blue	0.2ml

4% Paraformaldehyde Solution

4g of paraformaldehyde was dissolved in 100ml of PBS and the mixture was heated to 65°C whilst being stirred continuously. Once dissolved, the solution was made alkaline by the addition of 2 drops of 2M NaOH. The solution was then chilled to 4°C on ice and used immediately.

PCR Buffer

Tris-HCL pH 8.8	45mM
(NH ₄) ₂ SO ₄	11mM
MgCl ₂	4.5mM
Each dNTP	200μM
BSA	110μg/ml
β-mercaptoethanol	6.7mM
EDTA pH8.0	6.7mM

Appendix 2: Ethics Committee Approval

Melanie Sursham
Direct Dial 0116 2588610



LEICESTERSHIRE HEALTH
Gwendolen Road, Leicester LE5 4QF
Tel: (0116) 273 1173 Fax: (0116) 258 8577
DX 709470 Leicester 12

24 December 1996
Mr A R Naylor
Consultant Surgeon
Clinical Sciences Building
PO Box 65 The Leicester Royal Infirmary

Dear Mr Naylor

The role of Matrix Metalloproteinases in mediating carotid plaque morphology - our ref. no. 4454

Further to your application dated 22 October, you will be pleased to know that the Leicestershire Ethics Committee at its meeting held on the 6 December, 1996 approved your request to undertake the above-mentioned research. In granting this approval, I was asked to draw your attention to an article in the British Medical Journal (1996) 313:1366-8 as a result of which the Committee recommended that the consent forms be altered to indicate to patients that tissue may be removed for research purposes.

I would remind you, however, that your research project has been given approval only in relation to its acceptability from an ethical point of view. If, subsequently, departure from the methodology outlined in your protocol is contemplated, the Ethics Committee must be advised in order that the proposed changes may be approved. Also a report should be made to the Ethics Committee if any significant adverse reactions are noted during the course of the study. In addition, any NHS resource implications of your project must be discussed with the appropriate Trust Chief Executive. Similarly, it may be that the research project has implications for other disciplines and, if so, you are advised to discuss them with the appropriate departmental manager. Researchers should also be able to assure the Ethics Committee that satisfactory arrangements have been made for the labelling, safe storage and dispensation of drugs and pharmaceutical staff are always willing to provide advice on this.

Researchers' attention is also drawn to correspondence from the Regional Director of Public Health dated 28 January, 1991 relating to Clinical Trials which sets out revision of the procedures to be followed, and the Clinical Trials Indemnity Letter and Deed of Guarantee. Researchers should ensure that these indemnity arrangements have been complied with.

Researchers intending to study selective groups of patients in the community are reminded that their first approach should be to the individual patient's general practitioner to ascertain whether the particular patient was suitable for inclusion in the study. Equally, when the researcher contacts the patient it should be emphasised that the approach is made with the knowledge of the General Practitioner, with whom the patient may discuss this research, if the patient so wished.

Yours sincerely

M. Sursham

for Director of Public Health

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