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Extracellular matrix metabolism in injury-induced atherosclerosis

by

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A thesis submitted to the faculty of Graduate Studies and Research

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Abstract

Remodelling of extracellular matrix (ECM) is a prominent feature of atherosclerotic lesions and contributes to lipoprotein retention as well as smooth muscle cell (SMC) activation. To gain further knowledge about ECM, certain ECM components and their degrading enzymes were studied in injury-induced arterial neointima, which shares features with early atherosclerotic lesions.

It has been shown that synthesis of collagen and syndecan-1, a hybrid heparan/chondroitin sulfate proteoglycan, is enhanced. *In situ* hybridization indicates that syndecan positive cells are restricted to the arterial neointima. These data confirm the importance of arterial SMC in ECM metabolism and indicate that increased synthesis contributes to ECM accumulation in neointima.

Remodelling of ECM in atherogenesis refers not only to increased ECM deposition, but also involves enhanced ECM catabolism. A family of zinc-containing proteinases, termed matrix metalloproteinases (MMPs) has recently been implicated in atherosclerosis. Subsequently, we examined expression of two common MMPs, MMP-2 and MMP-9 in our model. The mRNAs for both MMPs are up-regulated, but their tissue distribution is different: MMP-2 positive cells are visible in neointima and in aortic media; whereas cells positive for MMP-9 are located only in the neointima. MMPs are active at neutral pH and in tissue, their activity is regulated by tissue inhibitors of metalloproteinases (TIMPs) including TIMP-1. The enhanced MMP expression in neointima makes it relevant to examine the simultaneous expression of TIMP-1. To do this, we cloned rabbit TIMP-1 from neointima using a PCR-cloning technique. Transformation of the cloned gene resulted in synthesis of a TIMP-1 protein in *E. Coli*. The concentration of TIMP-1 in the neointima was examined and a significant increase of both mRNA and protein levels was observed. It is suggested that the proteolytic activity of MMPs contributes to ECM breakdown. However, this digestion is limited, as continuous augmentation of TIMP-1 expression is observed after aortic de-endothelialization.

Résumé

Le remodelage de la matrice extracellulaire (MEC) est une caractéristique fondamentale des lésions d'athérosclérose et contribue à la rétention des lipoprotéines ainsi qu'à l'activation des cellules musculaires lisses (CML). Certains composants de la MEC et enzymes de dégradation ont été étudiés dans la neointima artérielle induite par abrasion, qui mime les caractéristiques des lésions précoces de l'athérosclérose.

Une synthèse accrue de collagène et de syndécan-1, un protéoglycan hybride d'héparan sulfate et de chondroitine sulfate a été démontrée. L'hybridation in situ indique que les cellules positives pour le syndican sont restreintes à la neointima artérielle. Ces données confirment l'importance des CML artérielles dans le métabolisme de la MEC et montrent qu'une synthèse accrue contribue à l'accumulation de l'MEC dans la neontima.

Le remodelage de la MEC au cours de l'athérogénèse consiste non seulement en une déposition accrue, mais implique également un catabolisme de la MEC. Une famille de protéases contenant du zinc, appelées métalloprotéases de la matrice (MPM) a récemment été impliquée dans l'athérosclérose. Nous avons examiné dans notre modèle l'expression de 2 MPM, MPM-2 and MPM-9. Les ARNm des 2 MPMs sont augmentés, mais leur distribution cellulaire est différente: les cellules MPM-2 positives sont localisées dans la neointima et la media aortique alors que les cellules MPM-9 positives sont localisées uniquement dans la neointima. Les MPMs sont actives à pH neutre et dans les tissus. Leur activité est régulée par les inhibiteurs des métalloprotéases (IMPT) y compris IMPT-1. A cause de l'expression augmentée de MPM dans la neontima, il est important d'examiner l'expression simultanée de l'IMPT-1. Dans ce but, nous avons cloné l'IMPT-1 à partir de la neointima aortique du lapin en utilisant une technique basée sur le PCR. La transformation de bactéries E. Coli dans un système d'expression a résulté dans la synthèse de la protéine IMPT-1. La concentration d'IMPT-1 dans la neointima a été étudiée et nous avons trouvé une augmentation significative de l'ARNm et de la protéine. Nous suggérons que l'activité protéolytique des MPMs contribue à la dégradation de la MCE. Cependant, cette dégradation est partielle, en raison d'une augmentation persistante de l'IMPT-1 après la dé-endothélialisation de l'aorte.

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TABLE OF CONTENT

Abbreviations

CHO: Chinese hamster ovary

CS: chondroitin sulfate

DS: dermatan sulfate

ECM: extracellular matrix

EDRF: endothelium-derived relaxing factor

EGF: epidermal growth factor

FGF. fibroblast growth factor

GAG: glycosaminoglycan

HA: hyaluronic acid

HS: heparan sulfate

IFN: interferon

IL: interleukin

LDL: low density lipoprotein

LP: lipoprotein

MMP: matrix metalloproteinase

PCR: polymerase chain reaction

PDGF: platelet-derived growth factor

PG: proteoglycan

PGI_2: prostacyclin

rER: rough endoplasmic reticulum

SMC: smooth muscle cell

TGF: transforming growth factor

TIMP: tissue inhibitor of metalloproteinases

TNF: tumour necrosis factor

TxA_2: thromboxane A_2

Prologue

Strategy of research

Atherosclerosis is a complex disease. Its development involves not only lipid and cellular components, but also various ECM components, including collagen, proteoglycan, elastin, and structural glycoproteins. The active role of ECM components in atherogenesis has recently been recognized, and their potential importance is the focus of much current research. This dissertation, in continuation with the long term research endeavor of the laboratory that I have joined for my PhD training, addresses the metabolic changes (remodelling) of arterial ECM in early atherosclerotic lesions. The model employed is rabbit aortic neointima, developed in response to a balloon catheter-induced deendothelialization.

I started my program by assessing the synthetic alteration of ECM in aortic neointima. Since collagen and proteoglycan constitute the major ECM components and take part in LP retention, they were chosen for study. The metabolic alteration of collagenous protein and mRNA expression of collagen type I, type III, and syndecan-1, a member of a family of integral membrane PG were measured. Syndecan-1 has also been suggested to act as a cell surface receptor for ECM via its interactions with extracellular ECM components and cytoplasmic actin. It is known that SMC migration and proliferation are important cellular features of atherosclerosis and several recent studies suggest that ECM degradation via MMP activity is a pre-requisite. Consequently, I examined the time-course of expression of mRNA for two common MMPs, MMP-2 and MMP-9, by Northern blot analysis and *in situ* hybridization. It is known that MMP

activity is regulated extracellularly by their inhibitors. A major family of MMP inhibitors are TIMPs, including TIMP-1. Therefore, I investigated expression of neointimal TIMP-1 by PCR-based cloning, Northern Blot, Western Blot, and mRNA *in situ* hybridization technique.

Rationale of Presentation

This thesis is presented in a manuscript style. A general introduction covering the literature on related studies is presented, followed by four chapters:

CHAPTER 2: Deendothelialization modulates collagen biosynthesis of arterial smooth muscle cells

CHAPTER 3: Expression of syndecan-1 by rabbit neointimal smooth muscle cells

CHAPTER 4: Expression of mRNA for metalloproteinases and their inhibitor by rabbit neointimal smooth muscle cells

CHAPTER 5: Synthesis of tissue inhibitor of metalloproteinases-1 in rabbit aortic neointima after selective deendothelialization

All these chapters have been submitted as original articles for publication. A hypothesis is put before every chapter serving as a logical bridge between chapters. In the final part of the dissertation, an overall conclusion and original contributions made from these studies are presented.

The experiments described in this thesis did not reproduce earlier works and represent original contributions to the field of atherosclerosis research. Except for the analysis of collagenous protein (which was done by Dr. Zhihe Li), I proposed the issues to be studied in my project and the experimental strategy to be employed. The work contained in this thesis was possible due to the guidance of my supervisor, Dr. M.Z. Alavi and my advisor, Dr. Sean Moore.

Dr. Alavi and Dr. Moore have initially pointed out the most relevant aspects related to this model of atherosclerosis and afterwards have frequently discussed the results of my experiments. They also provided me with a lot of literature on the subject. Also they helped me to get integrated into the larger project that is under study in the laboratory.

CHAPTER 1: INTRODUCTION

1.1 Atherosclerosis

1.1.1. Definition and background

Atherosclerosis is a disease of medium- and large-sized musculo-elastic arteries, and its complications in coronary and cerebral arteries are the leading cause of death in Western societies (Averbook *et al.*, 1989). Fully developed atherosclerotic lesions usually consist of two components: first, a central lipid pool which is formed predominantly by such lipids as free cholesterol and cholesterol ester; second, surrounding fibrous tissue which contains SMC and ECM components: including collagen, PG, elastin and structural glycoproteins (Wissler, 1992).

The pathological study by Ruffer (1911) indicates that atherosclerosis was prevalent even in ancient Egypt. But our ancestors usually consider it as a natural process of aging. It is believed that investigation of atherosclerosis as a disease started with Rudolf Virchow in 1856 (Long, 1967). Over the years, many hypotheses have been proposed to explain the pathogenesis of the disease. Until 1970s, the blood "imbibition", lipid "insudation" and thrombus "encrustation" hypotheses were widely used to explain the pathogenesis of atherosclerosis (Long, 1967). No doubt these hypotheses led to ways to improve our understanding of the disease, but many aspects of the disease remained ambiguous. In 1973, Moore provided the first experimental evidence that injury to aortic endothelium can induce atherosclerosis (Moore, 1973). Three years later, Russell Ross proposed the "response to injury" hypothesis (Ross and Glomset, 1976). More recently, Steinberg *et al.* (1989) stressing the crucial role of oxidized lipoprotein in

the initiation and development of atherosclerotic lesions, proposed that atherosclerosis is a complex disease in which many factors act in concert to bring about pathological changes. Endothelial injury or dysfunction may indeed be the underlying mechanism . However, the existing high mortality rate of atherosclerosis reminds us that more work is necessary to understand this complex disease.

1.1.2. **Normal arterial wall and atherosclerotic lesion**

1.1.2.1. **Structure and function of arterial wall**

Arteries generally have three basic layers or tunicae: an inner *tunica intima*; an intermediate *tunica media*; and an external *tunica adventitia*. According to their size and certain histological features, arteries are classified into large, elastic arteries; medium-sized, muscular arteries; and small, arterioles. The first two are major sites of atherosclerotic lesions, and the following discussion will focus on their structure (Moore, 1995).

Intima

Although barely visible in many vessels, the tunica intima in certain segments of the aorta is quite obvious (Badimon *et al.*, 1993). In these areas, the intima is usually composed of: 1) a monolayer of polygonal endothelial cells, often hard to identify by routine histological examination; 2) a very thin basal lamina; 3) a subendothelial layer, composed of reticulated PG, collagen, elastic fibrils, as well as SMCs; and 4) internal elastic lamina, regarded both as part of the intima and the beginning of the media (Stary *et al*, 1992).

The intima, especially the endothelial cells, plays a key role in regulating various arterial functions in health and disease. First, endothelial cells form a selective barrier between blood constituents and arterial tissue (Renkin, 1988). This property is responsible for the transport of

vascular nutrients and limited entry of lipoprotein into the vessel. Both transcytosis and passage through intercellular junctions are probable pathways for these macromolecules. Second, endothelial cells prevent deposition of platelets and formation of thrombi on arterial walls. The anticoagulant property of the endothelial cell is mediated by its synthesis of prostacyclin, plasminogen activator C, and by its binding with thrombomodulin, activated protein C, and plasminogen (Severs and Robenek, 1992). Third, endothelial cells are important in regulating vascular tone, mediated by their synthesis and release of such factors as: angiotensin II, endothelin, and EDRF (Dzau, 1994).

Media

Tunica media constitutes the major part of the arterial wall of elastic and muscular arteries. It is bounded inwardly by the internal elastic lamina and outwardly by the external elastic lamina. The media in elastic arteries consists chiefly of thick, fenestrated elastic tissue with intervening layers of SMCs as well as ECM components. In muscular arteries, the media consists predominantly of elongated, spindle-shaped SMCs arranged in a circular configuration (Severs and Robenek, 1992).

The SMC is the only cell type in the arterial media. Early in life, the primary function of SMCs is to proliferate and to synthesize ECM components, and thus the formation of the vessel wall. As the vessel reaches its final size, the SMCs turn into highly specialized cells functioning to maintain arterial tone (Thie, 1992). In keeping with the multiplicity of their functions, SMCs are capable of expressing a wide range of phenotypes. At one end of the spectrum of phenotypic expression is the SMC whose function is almost exclusively that of synthesis; the cytoplasm is rich in rER, Golgi apparatus and free ribosomes, but myofilament bundles are scarce. At the other end

of the spectrum is the SMC that performs a contractile function. The cytoplasm of the "contractile" cells is filled with myofilment bundles, cytoplasmic organelles such as rER, Golgi apparatus and ribosomes are few in number and located in the perinuclear regions (Li *et al*, 1994). In pathological states, "contractile" SMCs can be de-differentiated and express a synthetic phenotype, accompanying excessive production of ECM components (Davies and Woolf, 1993). In atherosclerotic plaques, SMCs express a variety of phenotypes ranging between synthetic and contractile extremes (Campbell and Campbell, 1987).

Adventitia

The adventitia is the outmost layer of the arterial wall. It usually starts from the external elastic lamina and blends into the connective tissue around vessels. Its thickness varies considerably, depending on the type and location of the vessels. The cerebral arteries are usually devoid of this layer (Badimon, 1993). Adventitia usually contains 1) collagen and elastic fibres, existing in irregular configuration; 2) lymphatic capillaries, draining fluid and macromolecules; 3) autonomic nerve fibres, regulating the tensile strength of the vessel; and 4) vasa vasorum, providing nourishment to the outer part of the media (Moore, 1996).

Although the lesion of atherosclerosis is principally present in the arterial intima, investigators have suggested that abnormalities of the vasa vasorum, resulting in poor blood supply to the tunica media, are important in atherogenesis. The "anoxic" hypothesis proposed that occlusion of vasa vasorum will promote hypoxia of the outer media and induce release of cytokines as well as other growth factors from arterial cells. These mitogens subsequently activate SMCs and trigger cellular proliferation and migration. In addition, hypoxia can upregulate activity of acyl Co A: cholesterol acyltransferase (ACAT) to enhance cholesteryl ester

formation and lipid deposition (Baker and Martin, 1994).

1.1.2.2. **Atherosclerotic lesions**

Atherosclerosis is a chronic disease and its lesions are heterogenous. In a particular individual, the abdominal aorta is usually involved earlier and more extensively, followed by coronary arteries, thoracic aorta, femoral arteries, internal carotid arteries, and vertebral arteries (Wissler, 1992). Within the same artery, there are also definite lesion-prone and lesion-resistant sites. For example, in carotid artery, the more advanced plaques usually involve the bifurcation area, especially the crotch (Thubrikar and Robicsek, 1995). A number of distinct atherosclerotic lesions have been described and recently classified into early (type I, type II, and type III) and advanced (type IV, type V and type VI) lesions by Stary *et al.*, (1995).

Type I lesions

Type I lesions occur in the initial stage of atherosclerosis, consisting of minimal lipid deposits that may not be visible to the naked eye. Under the microscope, however, isolated foam cells with intracellular lipid droplets are visible. The type I lesion is usually seen in infants and children. But this lesion may also be found in some adults, particularly in arterial regions that are regarded as lesion-resistant (Stary *et al.*, 1994).

Type II lesions

Type II lesions may appear as yellow streaks, patches, or spots on the arterial intima. Microscopically, focal accumulation of foam cells is seen. Both SMCs and macrophages contribute to foam cell formation (Moore and Alavi, 1995). While most lipid of type II lesions resides within the cells, small quantities of lipid droplets are also distributed in the extracellular space. Biochemically, lipid in this lesion consists primarily of cholesterol ester, free cholesterol,

and phospholipid.

Type II lesions are further divided into two groups. The lesions in one group are prone to advanced lesions and are called type IIa. The lesions in another group are termed type IIb, which either do not progress, progress slowly, or progress only in individuals with high levels of blood lipoproteins. Morphologically, type IIa lesions differ from type IIb lesions by their abundance of extracellular matrix components and SMCs, explaining their tendency for disease development (Williams and Tabas, 1995).

Type III lesions

The characteristic histological feature of type III lesions is the formation of one or more small extracellular lipid pools among layers of SMCs. Biochemically, lipid in this lesion contains more free cholesterol, fatty acid, sphingomyelin, lysolecithin, and triglyceride than type II lesions. The melting temperature of cholesterol ester droplets in this lesion is also lower than that of the type II lesion (Stary *et al.*, 1994)

Type IV lesions

The type IV lesion, also known as atheroma, shows a macroscopically visible lipid core with a well-defined border and calcium deposits. The organelles of neighbouring SMCs may also be calcified. The shoulder regions of atheroma are vulnerable to rupture because of the dense concentration of macrophages and lymphocytes. Also, capillary formation surrounding the lipid core may increase the vulnerability of the shoulder region (Libby, 1995).

Type V lesions

The definitive feature of the type V lesion is excessive formation of fibrous tissue and organization of thrombus remnant. The fibrous tissue consists of substantial amounts of

collagenous protein and SMCs rich in rER. Lymphocytes and macrophages are frequently observed in this region and small hemorrhages may also occur.

Type VI lesions

Type VI lesions refer to advanced lesions with superimposed complications. Type VI lesions are subdivided according to the superimposed features. Disruption of the surface, including fissure and ulceration, may be termed type VIa; hematoma or hemorrhage, type VIb; and thrombosis, type VIc. Type VIabc indicates the presence of all three features (Stary, 1995). Clinically, these lesions often cause occlusion of arteries that can result in tissue infarction. Type VI lesions may also be a source of vascular emboli.

Atherosclerotic aneurysms

Aneurysm formation is another common complication of atherosclerosis; it usually occurs in the advanced stage as a result of extensive destruction of the tunica media. The factors favouring aneurysm formation may include increased proteolytic digestion, local hemodynamic alteration and genetic predisposition. The rupture of an aneurysm is usually followed by massive and fatal haemorrhage (Moore, 1996).

1.1.3. **Pathogenesis of atherosclerosis**

Despite the clear description of atherosclerotic lesions, our understanding of its pathogenesis is still incomplete. However, most investigators are of the view that atherogenesis is an immune/inflammatory response of the arterial wall to endothelial injury (Davies and Woolf, 1993).

1.1.3.1. **Injury to endothelial cells**

Endothelial cells have a central role in the pathogenesis of atherosclerosis. The concept of

endothelial injury implies a spectrum of changes ranging from denuding of the entire lining to subtle functional alterations (Alavi et al., 1985; Ross, 1986). A wide variety of factors can induce injury, including severe hypertension, smoking, hyperlipidemia, homocysteinemia, immune complexes, viruses and a variety of mechanical devices (Severs and Robenek, 1992).

The injured endothelial cells have several properties favouring atherogenesis. First, these cells can secrete growth factors, such as PDGF, which stimulate migration and proliferation of arterial SMCs (Ross, 1992). Injured cells can also synthesize other macromolecules to regulate SMC proliferation. For example, they synthesize angiotensin II and thromboxane A_2 to stimulate SMC proliferation; and they produce EDRF (NO), and prostacyclin to inhibit SMC proliferation (Dzau, 1994). Second, injury alters the nonthrombogenic properties of endothelium, leading to platelet attachment and formation of mural thrombi (Stehbens, 1992). The aggregated platelets secrete growth factors for arterial SMCs (see below). Third, injury can increase endothelial permeability, permitting increased penetration of plasma lipoproteins and other macromolecules, such as fibrinogen, into the vessel wall. The entry of the plasma components further enhances the extent of endothelial injury (Falk and Fernandez-Ortiz, 1995).

Injury to endothelial cells has recently been related to initiation of atherogenesis by lipoprotein, especially oxidized LDL. Oxidized LDL is cytotoxic to endothelial cells in vitro, inducing alterations ranging from abnormal gene expression to complete cell denudation (Witztum and Steinberg, 1991). The existence of oxidized LDL in human atherosclerotic lesions has recently been confirmed (Steinberg and Witztum, 1990). Endothelial cells, SMCs and macrophages can all oxidize lipoprotein in vitro and the reaction depends on the presence of trace metals. The oxidation induces a continuum of changes of lipoprotein related to the extent of

oxidization (Halliwell, 1995).

1.1.3.2. **Thrombosis in early atherogenesis**

Endothelial injury is usually followed by an immediate accumulation of platelets on the damaged areas and the platelets subsequently degranulate (Fuster *et al.*, 1991). The platelet deposition may be related to functional alteration of endothelial cells, i.e., loss of non-thrombogenic activity; exposure of subendothelial ECM such as collagen; or due to injury-induced expression of tissue factors in arterial smooth muscle cells (Taubman, 1993). The accumulated platelets will release a battery of mitogens, including PDGF, FGF, TGF-α and TGF-ß (Ross, 1992). If the injury is severe enough or repeated, increased production of thrombin will occur (Loscalzo, 1992). These mitogens and thrombin take a critical part in SMC migration and proliferation. PDGF is an important mitogen for arterial SMCs, as confirmed by the blocking of SMC proliferation using PDGF neutralizing antibody (Ferns *et al*, 1991). PDGF exerts its action through specific cell surface receptors (α- and ß-receptors). Binding of PDGF with its receptors leads to formation of homo- or heterodimers of receptors which are then phosphorylated. Subsequent signal transduction pathways have not been fully identified, but may relate to *ras* protein and intracellular calcium (Eriksson *et al.*, 1992). The net result of the signalling is to move SMCs from G_0 to G_1 phase (Badimon *et al*, 1993). The importance of thrombin in atherosclerosis has been suggested by the presence of its receptor in almost all cells involved in the disease (Falk and Fernandez-Ortiz, 1995). Recent studies indicate that thrombin can influence SMC proliferation and migration (Wilcox, 1993). The intracellular signalling may involve G protein mediated- and tyrosine kinase mediated-pathways (Baykal *et al.*, 1995). Fibrinogen and fibrin degradation products (FDP) may be another linkage between thrombosis and

atherosclerosis. Epidemiological studies indicate that fibrinogen is an independent risk factor for atherosclerosis (Kannel, 1987). Fibrinogen is important in extracellular lipoprotein retention, as the colocalization of fibrinogen and LDL/apolipoprotein B has been revealed in atherosclerotic plaques (Beisiegel, 1990). In addition, fibrinogen and FDP can also induce SMC migration and proliferation (Loscalzo, 1992). Immunological approaches identify the presence of fibrin(ogen)-related antigen in early atherosclerotic lesions (Zhang *et al*, 1993).

1.1.3.3. **Arterial SMC**

Numerous studies of human as well as experimentally induced atherosclerosis have shown that SMCs not only constitute the principal cellular component in the lesion, but also exert multiple functions in accelerating atherogenesis (Haust and More, 1960; Wissler, 1991). In injury-induced atherosclerosis of rabbits, Moore and his colleagues (Moore, 1971; Alavi and Moore, 1985) have repeatedly shown that SMC migration and proliferation appear to be the initial response to endothelial injury. The activation of SMCs leads to the formation of a neointima, an early lesion of atherosclerosis. About 50% of intimal SMCs are in the proliferative state. Similarly, Schwartz and Reidy (1987) have shown that SMC proliferation occurred immediately after a massive loss of endothelium in the rat. Platelet-derived growth factor (PDGF) is important for SMC activation, as depletion of platelets dramatically reduces the mitogenic response of SMCs to arterial injury (Moore *et al.*, 1976). On the other hand, SMC proliferation may also be due to the absence of inhibitory agents, such as heparin-like molecules and laminin (Schwartz and Reidy, 1987). Over the years, many more factors have been identified, predominant among them are TGF-ß, FGF, PGI_2, and EDRF. These factors are secreted by platelets, endothelial cells, macrophages, T-lymphocytes and SMCs (Desmouliere and Gabbiani,

1992). In advanced atherosclerotic lesions, oxidatively modified lipoprotein can also induce migration and proliferation of SMCs (Witztum and Steinberg, 1991).

It has been found that proliferating SMCs in atherosclerotic lesions synthesize large amounts of ECM components, including collagen, PG and fibronectin (Alavi and Moore, 1987; Raines and Ross, 1992). Three months after injury, extracellular matrix comprises more than three quarters of the neointima (Reidy, 1985). ECM components not only contribute to the occlusive nature of atherosclerosis, but are also involved in thrombosis, lipoprotein retention and SMC activation.

1.1.3.4. **Lipid deposition in atherogenesis**

Water-insoluble lipids are transported in blood in the form of plasma lipoproteins (LPs) which are spherical molecular complexes composed of apoprotein and lipids. According to their density as well as composition, LPs are divided into five families: chylomicrons (< 0.95 g/dl); very low density lipoproteins (VLDL=0.95-1.006 g/dl); intermediate density lipoproteins (IDL= 1.006-1.019 g/dl); low density lipoproteins (LDL= 1.019-1.063 g/dl); and high density lipoproteins (HDL= 1.063-1.21 g/dl). A recently identified lipoprotein is lipoprotein (a), which is structurally similar to LDL but contains an additional apolipoprotein called apolipoprotein (a) [LP(a)] (Ginsberg, 1994).

Most lipids deposited in atherosclerotic lesions are derived from plasma LDL. LDL carries about 60-70% of the total plasma cholesterol and its protein component is primarily apolipoprotein B (O'Brien and Chait, 1994). The intravascular accumulation of cholesterol seems to be regulated by two different mechanisms, one is active and the other is passive. The active LDL intake is dependent on specific receptors on the cell surface. For example, LDL

accumulation in macrophages is primarily mediated by the scavenger receptor and the B-VLDL receptor; and its accumulation in SMCs is largely regulated by LDL (apo B/E) receptor (Mahley, 1988; Dejager *et al.*, 1993). The passive retention of LDL is closely related to the function of the extracellular matrix.

1.1.3.5. **Plaque disruption**

Fissuring or rupture of atherosclerotic plaques usually occurs in advanced atherosclerosis and is related to acute ischemic syndromes, such as myocardial infarction (Moore, 1996). Plaques prone to rupture usually have less collagenous content in the fibrous cap and a large, eccentric lipid pool (Libby, 1995). Two factors seem decisive for plaque rupture: 1) the force acting on the plaque; 2) the tensile strength of the plaque (MacIsaac *et al.*, 1993). Emotional stress and physical activity, two common initiating events of ischemic syndromes, can raise coronary blood pressure and thus increase the force on coronary atherosclerotic plaques. Turbulent flow caused by the plaque itself may also contribute to excessive force on the plaque (Thubrikar and Robicsek, 1995). Considering the tensile strength, rupture tends to happen at the junction between the fibrous cap of the plaque and adjacent normal tissue, where the plaque is extensively infiltrated by macrophages and T lymphocytes (Lendon *et al*, 1991). The cytokines secreted by the inflammatory cells influence ECM metabolism. Cytokines like IFN-gamma inhibit the synthesis of collagens that govern the integrity of the fibrous cap (Libby, 1995). On the other hand, IL-1, TNF and PDGF stimulate the synthesis of MMPs and help to activate them (Mauviel, 1993). The MMPs can digest ECM components and decrease the tensile strength of the atherosclerotic plaque cap.

1.1.3.6. **Thrombosis in advanced atherosclerosis**

Thrombosis is a common, and sometimes fatal complication in advanced atherosclerosis. Intracoronary thrombi have been found in 70% of cases of sudden cardiac death (Falk, 1991). In fact, it has been proposed that atherosclerosis is generally a benign disease in the absence of thrombosis (Falk and Fernandez-Ortiz, 1995). The initial event of the thrombotic response is platelet aggregation which is reinforced by the progressive polymerization of fibrin from fibrinogen. Occlusive thrombi usually develop on the basis of plaque rupture and develop from within the plaque.

The thrombotic response to plaque rupture is dependent on 1) features of exposed thrombogenic materials; 2) local flow characteristics; and 3) systemic thrombogenic factors. One study has been performed to compare the thrombogenicity of material from human atherosclerotic lesions, including that from type II, type III, Type IV, type V lesions. It was found that the lipid core in atheromatous lipid-rich plaques is most thrombogenic, approximately 6-fold more than the collagen-rich sclerotic components (Fenandez-Ortiz *et al.*, 1994). Thrombus formation also increases with increasing degrees of luminal stenosis, probably due to platelet activation in high shear conditions. It has been suggested that the thrombotic response after plaque disruption depends, at least in part, on the sudden geometric changes at that site (Lam *et al.*, 1994). Systemic factors are also important in thrombus formation in atherosclerotic lesions as well. In this regard, it has been reported that catecholamines, cholesterol, and diabetes can enhance platelet coagulation activity (Fuster, 1994). Increased activity of the renin-angiotensin system relates to acute coronary syndromes by interfering with the fibrinolytic system (Van Leeuwen *et*

al., 1994).

1.1.4. **Experimental atherosclerosis**

Atherosclerosis is a complex process. A variety of animal species has been employed in its study (Russel, 1995). Whatever the approaches are, it is generally agreed that an ideal model should reproduce atherosclerotic lesions similar to human atherosclerosis morphologically, topographically, and biochemically. As a consequence, lesions in the model would range from mild abnormalities such as fatty streaks, to raised plaques with complications such as ulceration, intraplaque haemorrhage, and superimposed thrombosis (Clarkson *et al.*, 1987).

1.1.4.1. **Diet-induced atherosclerosis**

Cholesterol-enriched food is widely used in atherosclerosis-induction in animals. Nonetheless, other regimens such as diet containing high complex carbohydrates and diet-induced hypervitaminosis D have also been related to experimental atherosclerosis. The first rabbit model of atherosclerosis was established by feeding diets composed of milk, meat and eggs (Ignatovski, 1908). But this change was attributed to the injurious effect of animal protein. It was Anitschkow (1912) who produced atherosclerosis in rabbits by feeding them pure cholesterol, dissolved in vegetable oil. Later on, this approach has been applied in the induction of atherosclerosis in pigeons, pigs, chickens, monkeys and baboons (Long, 1967). However, the high cholesterol content in these diets causes much higher plasma cholesterol levels than those usually occuring in atherosclerotic patients. Wilson *et al.* (1982) successfully induced atherosclerosis in rabbits using a diet of cholesterol and fat levels comparable to that of North Americans. The lesions produced contained a lipid pool, a fibromuscular cap, a large number of macrophage-derived foam cells and some proliferating SMCs. The diet-induced model helps to clarify the different roles of saturated

and unsaturated fat in atherogenesis; exhibits the potential regression of atherosclerotic lesions; and benefits our understanding of the cellular interactions in atherogenesis (Armstrong *et al.*, 1970; Richardson *et al.*, 1989). However, one must be cautious in interpreting data generated by studies employing this model, because effects vary significantly using different dietary regimens.

In certain animals, lesions induced by high cholesterol, or high fat diets are composed primarily of macrophage-derived foam cells with little extracellular lipid; the fibrous cap and complications of atherosclerosis may not be present (Clarkson *et al.*, 1987). Topographically, proximal coronary arteries and cerebral arteries are usually spared of disease; in contrast, there is abundant lipid deposition throughout the reticuloendothelial system, such as liver spleen and bone marrow (Prior *et al.*, 1961). These lesions bear a close resemblance to human lipid storage disease (Clarkson *et al.*, 1987).

1.1.4.2. **Injury-induced atherosclerosis**

The idea that atherosclerosis is the response of the arterial wall to injury originated from early observations of Virchow in the nineteenth century (Long, 1967). However, the suggestion that endothelial injury is related to atherogenesis in the absence of hyperlipidaemia was made by Duff (1936) and later by Haust and More (1965). Early attempts to induce atherosclerosis in animals by injury often resulted in a fibro-musculo-elastic plaque due to SMC proliferation (Moore, 1981). In the early 1970s, it was for the first time shown that arterial injury in normolipidemic rabbits can produce lipid-containing lesions (Moore, 1971; 1976). In these experiments, a continuous intimal injury by an indwelling aortic catheter was used. Later on, it was found that a single removal of endothelium will produce similar lipid-rich lesions.

From that time, various types of manipulations, including balloon catheter de-

endothelialization, X-ray irradiation, air-drying and immunological damage, have been applied to induce lesions (Severs and Robenek, 1992). But balloon catheter deendothelialization, a technique originally described by Baumgartner, remains the method of choice for most researchers. Basically, this approach consists of drawing a partially inflated Fogarty embolectomy catheter through a vessel. This will allow the selective removal of the endothelium, with minimal damage to the underlying tissues. The primary reaction following the removal of endothelium is the formation of a monolayer of platelets, even though interaction between leukocytes and subendothelial tissue also increases. The platelet adherence is short-lived and polymerized fibrin is not observed (Richardson *et al.*, 1989). Four days after deendothelialization, development of the neointima in injured areas is visible. The neointima consists primarily of proliferating SMCs and ECM components.

When vessel injury is induced, concomitant with administration of an atherogenic diet, an advanced lesion will develop within a short period of time, indicating a synergistic relationship between injury and diet (Alavi *et al.*, 1991).

1.1.4.3. **Genetic models for atherosclerosis**

Advances in molecular biology have fostered powerful approaches to establish genetic animal models for the study of atherosclerosis. These models not only enhance our capacity to study the influence of inherited factors on this vascular disease, but also provide an opportunity to evaluate the functions of various genes in vivo (Lusis, 1993). The current strategies to generate genetic models include: inbred genetic strains, transgenic animals, gene targeting by homologous recombination and in vivo gene transfer.

Inbred genetic animal models

The most notable model in this category is the Watanabe heritable hyperlipidemic (WHHL) rabbit. Rabbits in this strain harbor a genetic defect in their LDL receptor gene, identical with that in patients suffering from familial hypercholesterolemia. The reported plasma cholesterol level in homozygous WHHL rabbits is from 650 to 950 mg/dl. Studies of WHHL rabbits provide valuable information on the relationship between LDL and atherosclerosis (Kita *et al.*, 1995). A similar approach has been adopted to characterize inbred strains of familial hypercholesterolemic (FHC) swine (Prescott *et al.*, 1995) and the JCR: LA-corpulent rat, both of which are predisposed to atherosclerosis (Russel, 1995).

Transgenic animal models

Transgenic technology involves the addition of a foreign gene (usually in the form of DNA) to germ line cells, by microinjection. The injected DNA, usually ligated end to end to form a tandem array, integrates randomly into oocyte genomic DNA. The manipulated oocyte will then be reimplanted into pseudopregnant female mice. Offspring that permanently express foreign genes are subsequently selected to homozygosity. Theoretically, this technique allows the evaluation of an individual gene. One successful application of this technique was performed by Hoffmann *et al.* (1988), showing that a high level of LDL receptor expression helps transgenic mice to rapidly clear plasma LDL. This experiment provides direct evidence for the role of the LDL receptor in lipoprotein metabolism.

In vivo gene transfer

All of the above techniques deal with gene modification in germline cells of animals. Recently, techniques have been developed to introduce foreign DNA into host somatic cells. When this manipulation is used for in vivo synthesis of missing or defective genes in patients with

inherited or acquired disorders, it is called gene therapy. The application of this technique in cardiovascular research has generated promising results. For example, it is well recognized that vascular SMC proliferation in response to injury is an important etiologic factor in vascular proliferative disorders. This proliferation is significantly inhibited by localized infection of arterial cells with adenovirus encoding an active Rb gene. The Rb gene is capable of producing a cell cycle progression inhibitor (Change *et al.*, 1995). Besides proving the importance of Rb in regulating SMC proliferation, these results indicate a potential therapeutic approach for restensosis following arterial injury.

1.2. Extracellular matrix (ECM) in arterial wall

1.2.1. Introduction to arterial ECM

ECM is composed of complexes that constitute a biological milieu in which most cells live and function. In the normal vascular wall, these macromolecules interact with each other and arrange themselves in a specific fibrous matrix which either surrounds SMCs, or remains in contact with endothelial cells at their basal side. Vascular ECM components include: 1) collagen; 2) elastin; 3) proteoglycans; 4) structural glycoproteins, such as fibronectin, laminin, and thrombospondin. (Hay, 1991). In the artery, both SMCs and endothelial cells produce ECM. However, the synthesis by SMCs is more prominent. ECM synthesis in the normal artery is highly regulated, in a spatial and temporal fashion (Tan and Uitto, 1989).

ECM components perform many functions: collagen and elastin regulate the tensile strength and elasticity, respectively; PGs control transvascular diffusion of macromolecules and facilitate cellular adhesion (Borel *et al.*, 1987). Recently, it has been revealed that ECM exert a profound effect on neighbouring cells, influencing cellular differentiation, migration and

proliferation (Hay, 1991; Moore and Alavi, 1995). It has been shown that interactions between ECM and cells are mediated by cell surface molecules. It is known that a small portion of PGs have transmembrane protein cores, the best known examples are CD44 and syndecans. Both CD 44 and syndecans can act as adjunct receptors by binding various ECM elements and help to transduce extracellular signals into the cell interior (Jalkanen *et al.*, 1991). The primary mediators of ECM signals are a family of cell surface receptors known as integrins. Integrins are a family of glycoproteins consisting of 2 subunits, α and ß, that are noncovalently bound to one another. So far, 11 types of α and 6 types of ß subunits have been identified. They, by various combinations, form at least 16 different classes of integrins (Ruoslahti, 1991 a). It is suggested that both subunits contribute to the formation of ECM ligand-binding sites and the ß-subunit is also responsible for intracellular signal transduction (Tapel *et al.*, 1989). The tripeptide RGD (or Arg-Gly-Asp) region of many ECM components is believed to be a principal binding site for integrin, although the sequences flanking or the conformation spanning this tripeptide may further regulate binding specifity (Ruoslahti, 1991 b). The ECM components, via their interaction with integrins, influence cellular behavior in many aspects. In a gene transfer experiment, CHO cells that express a high level of integrin $\alpha_5\beta_1$ attract more fibronectin and become less migratory in soft agar (Giancotti and Ruoslahti, 1990). Binding of ECM to integrins will also alter assembly of cytoskeletal structures, on which certain cellular mRNAs need to reside to be active. It has been proposed that alteration of cytoskeletal assembly is responsible for ECM-stimulated collagen synthesis in embryonic corneal epithelium (Sugrue, 1987). Some authors also suggested the participation of ECM-mediated events in transcriptional gene regulation, but the cytoplasmic pathway is unclear (Schmidhauser *et al.*, 1990).

1.2.2. Collagen

1.2.2.1. Collagen in arterial wall

Fifteen types of collagen have been identified. At least 5 types are present in blood vessels. Collagen comprises 20% of protein content in aorta and 40% of protein in smaller arteries (Tan and Uitto,1989). The arterial collagen is divided into 1) fibril-forming collagen; 2) basement membrane collagen; 3) microfibrillar collagen. The fibril-forming, or type I, III, and V, collagen occupies 80-90 % of the total collagen content in the blood vessel. Type I collagen is most abundant in amount and is highly resistant to mechanical stress. Type III collagen usually forms cross-banded fibrils with type I collagen, although the presence of type III collagen without type I has been observed in the intima of young individuals (Keene *et al*, 1987). The localization and organization of type V collagen in the vessel have not been fully established. However, the amino acid sequence of this collagen suggests features of fibril-forming interstitial collagen, and in vitro formation of hybrids of type I-V fibrils has been shown (Adachi and Hayashi, 1985). The basement membrane collagens refer to type IV, and possibly type VIII collagens (Rauterberg and Jaeger, 1992). Type IV collagen has been considered as a structural organizer of the basement membrane underlying the endothelial cells and surrounding the SMCs. Endothelial cells bind this collagen indirectly via laminin or fibronectin (Macarak and Howard,1983). Type VIII collagen was originally identified in cultured bovine aortic endothelial cells, and its presence in vivo remains an open question (Kapoor *et al.*, 1988). Microfibrillar, or type VI collagen distributes in all layers of blood vessels. It contains large globular domains in both terminals of peptide sequence. This collagen may play a distinct role in the assembly of the collagenous meshwork (Rauterberg and Jaeger, 1992).

The genes for collagen, especially for fibrillar collagen, usually have more than 50 exons. The majority of these exons are 54 bp or a multiple of 54 (108 or 162). Moreover, a significant portion of exons are multiples of 9 bp which encodes Gly-X-Y triplets, frequently X is proline and Y is hydroxyproline. Based on these observations, it has been proposed that ancestors of fibrillar collagen genes arise by amplification of a primordial gene consisting of a 54-bp exon unit (Yamada *et al.*, 1980).

Both biochemical and morphological studies reveal an elevated collagen content, accompanying an altered ratio between different types of collagen, in atherosclerotic arteries (Stiemer *et al.*, 1993). This increase primarily results from increased biosynthesis by intimal SMCs, occurring at both gene transcriptional and post-transcriptional levels. Rauterberg and Jaeger (1992) have shown remarkable difference in gene expression between normal and atherosclerotic arterial tissues. The pattern of gene expression may be due to the effect of growth factors and cytokines. In arterial SMCs, TGF-ß1 is the most efficient stimulator of collagen; PDGF modulates collagen synthesis as a secondary result of enhancing cell division; and IFN-gamma as well as bFGF suppress total collagen synthesis (Okada *et al.*, 1993). ECM components may also influence collagen metabolism. For example, contracted collagen lattice (containing mainly collagen type I and type III) strongly inhibited PDGF and TGF-ß induced collagen synthesis (Majors and Ehrhart, 1993).

1.2.2.2. **Role of arterial collagen in atherosclerosis**

Arterial collagen, especially type I and type III collagen, takes a major part in resisting mechanical stress and in limiting elastic deformation during systolic dilation. The global reduction of collagen content in arterial tissues, as in patients suffering from Ehlers-Danlos syndrome type

IV, makes an individual susceptible to aneurysm formation. The focal reduction of collagen content in atherosclerotic plaque favours plaque rupture (Tan and Uitto, 1989).

Excess accumulation of collagen in vessels, as seen in atherosclerosis, increases stiffness of arteries and may be related to lipoprotein retention (Miller *et al.*, 1993). Experimentally, Guyton and Klemp (1995) have observed that lipid accumulates as isolated vesicles, containing principally phospholipid and free cholesterol, within fibrillar collagen bundles. Jimi *et al.* (1994) showed that type I and type III collagen have a high affinity for LDL, especially oxidized LDL. A negative charge-dependent mechanism may be responsible for this binding.

Fibrillar collagen is also a substrate for platelet aggregation. Collagen binds to platelet surface integrin receptor-Ia/IIb, and thus influences platelet accumulation. When platelets adhere to collagen in vivo, they spread upon the fibril and release such factors as ADP, serotonin, von Willebrand factor, and PDGF. In addition, activated platelets also synthesize TxA_2 and PGI_2 which are potent regulators of vessel contraction and platelet aggregation (Kinlough-Rathbone and Mustard, 1987).

1.2.3. **Proteoglycans**

1.2.3.1. **Proteoglycans in the arterial wall**

Proteoglycans are complex molecules containing a protein core with one or more covalently attached GAG chains (Wight, 1989). A GAG chain is attached to core protein through a glycosidic bond between the reducing terminal sugar and the hydroxyl terminal of a serine and/or threonine residues in the peptide. The GAG chains are composed of repeating disaccharide units of an amino sugar (N-acetylglucosamine or N-acetylgalactosamine) and a uronic acid (glucouronic or iduronic). Four groups of GAGs are present in the arterial wall: 1)

hyaluronic acid; 2) chondroitin sulfate; 3) dermatan sulfate; 4) heparan sulfate and heparin. With the exception of hyaluronic acid, all these GAGs are present in the form of PGs (Bihari-Varga, 1987). Traditionally, PGs are classified according to their GAG chains. Thus CS-PG, DS-PG, and HS-PG, termed according to the most abundant GAGs, have been identified in the arterial wall. Recently, identification of PGs by their protein cores has been introduced, reflecting increased knowledge about protein content in PG.

1.2.3.2. **PG synthesis in atherosclerosis**

The observation of increased "mucous substances" in atherosclerotic lesions by Virchow in 1856 was probably the first to implicate PG in atherogenesis (Long, 1967). Later on, many investigators reported increased accumulation of PG in atherosclerotic arterial wall, as reviewed by Wight (1989) and Moore and Alavi (1995). This increase appears mainly to involve CSPG and DSPG, with minimal change in HSPG (Wight, 1989). It is known that accumulation of PGs in atherosclerotic lesions is predominantly the result of active synthesis by intimal SMCs (Li *et al.*, 1994).

Growth factors and cytokines as well as ECM components have an important role in regulation of PG synthesis. It has been reported that PDGF and TGF-ß1 can stimulate synthesis of versican, a large CSPG, by arterial SMCs and the stimulation by PDGF is inhibited by IL-1 (Wight *et al*, 1995). IL-1 and TGF-ß can up-regulate mRNA expression for decorin and biglycan, respectively (Edwards *et al.*, 1994). Both decorin and biglycan are small DSPG. ECM components may also affect PG synthesis. When arterial SMCs are cultured on hydrated collagen gel (primarily type I collagen), they decrease the overall PG synthesis, but increase the production of iduronic acid-rich Dermatan sulfate (Lark and Wight, 1986).

Besides protein content, structural alteration is also noted in the PGs of developing atherosclerotic plaque. CS- and DS-PG isolated from human atherosclerotic plaque tend to have large molecular masses (Berenson *et al.*, 1988). This alteration may also relate to the function of growth factors/cytokines. Both PDGF and TGF-ß1 cause elongation of GAG chains attached to PG core protein, thus increasing the overall size of these PGs (Wight *et al.*, 1995).

1.2.3.3. **Role of proteoglycans in atherosclerosis**

The PGs can influence cell proliferation in atherosclerosis. For example, HSPG can promote cell proliferation through binding with growth factors, such as bFGF, TGF-ß, and granulocyte-macrophage colony stimulating factor (Lortat-Jacob and Grimaud, 1992). It has been shown that bFGF bound to subendothelial HSPG is released slowly in active form, enhancing proliferation of endothelial cells (Gallagher, 1994). Binding with PG may also influence interaction between growth factors and their receptors. For example, binding with heparin or HSPG appears to be a prerequisite for ligation of FGF to its high-affinity receptor (Yayon *et al.*, 1991).

There is evidence that PGs can retain circulating lipoprotein by virtue of their avidity for LDL. More recently, co-localization of PG and lipoprotein in atherosclerotic lesions has been observed immunohistochemically. It has been suggested that interaction between PG and LDL is ionic in nature (Iverius, 1972). Three properties of the GAG appear to control its interaction with LDL: type of GAG chain; degree of sulfation of the disaccharide subunits; and the length of the chain (Jackson *et al.*, 1991). Evidence suggests that chondroitin sulfate and dermatan sulfate have the highest affinity for LDL (Bihari-Varga, 1987 b).

Binding with PG may significantly alter LP properties. It has been shown that LP-PG

complexes are taken up avidly by macrophages (Ismail *et al.*, 1994). LDL which is dissociated from LP-PG complexes, is bound, taken up and degraded more avidly by macrophages (Camejo. *et al.*, 1995). Binding with PG will also increase the residence time of LDL in the arterial wall and the opportunity for hydrolytic and oxidative modification of LDL (Williams and Tabas, 1995).

1.3. **Matrix metalloproteinases and their inhibitors**

1.3.1. **MMP and ECM degradation**

It is known that ECM components in arterial wall are not only being continuously synthesized, but also being catabolized. The ECM catabolism involves many proteinases, but the role of matrix metalloproteinases (MMPs) may be of critical importance (Davies *et al.*, 1992). At least nine MMPs have been identified and are divided into three subclasses: collagenase (MMP-1, MMP-8), gelatinases (MMP-2, MMP-9), and stromelysins (all other MMPs). The cellular sources of MMPs in the artery and their known substrates are listed in table 1.

Major MMPs and their properties in arterial wall

	MMP-1 (Collagenase)	MMP-2 (Gelatinase A)	MMP-3 (Stromelysin)	MMP-9 (Gelatinase B)
Potential Source				
SMC	+	+	+	+
Endothelial Cell	+	+	+	+
Macrophage	+	+	+	+
Fibroblast	+	+	+	+
Substrate				
Collagen I	+	-	-	-
Collagen III	+	-	+	-
Collagen IV	-	+	+	+
Gelatin	Weak	+	+	+
Proteoglycan	-	-	+	-
Fibronectin	-	-	+	-

MMPs are a family of structurally homologous proteinases, which are dependent on zinc for their activity (Wossener, 1991). The members of the MMP family share certain features: 1) they are secreted in an inactive form and become activated after limited proteolysis; 2) metal chelating agents such as EDTA and phenanthroline can block their activity; 3) the blockage by chelating agents can be reversed by adding exogenous Zn^{++}; 4) MMP activity is inhibited by tissue inhibitors of metalloproteinases (Newby *et al.*, 1994). Collectively, MMPs can digest almost all ECM proteins at neutral pH. Structurally, MMPs show a similar domain structure, which contains: 1) a signal peptide domain; 2) a propeptide domain; 3) a catalytic domain; and 4) a COOH-terminal domain (except MMP-7). Gelatinases have additional domains inserted into the

catalytic domain.

The propeptide domain is responsible for maintaining the latent state of MMPs. A highly conserved sequence in the propeptide domain, PRCGV/NPD, has been related to this function. (Springman *et al.*, 1990). The "cysteine switch" mechanism suggested that coordination of Cys residue in the conserved sequence to zinc atom in the catalytic domain is essential to keep MMP latent. The activation starts with physical or biochemical breakage of this coordination which permits further proteolysis of the C-terminal side of PRCGV/NPDV. The proteolysis leads to the formation of active enzyme. Park *et al.*, (1991) have shown that mutation of the Cys or the Arg residues will result in autoactivation of MMPs. And Steller-Stevenson *et al.*, (1991) indicated that synthetic peptide based on the PRCGV/NPDV sequence can inhibit MMP activity .

Because the catalytic activity of MMPs depends on Zn, much effort has been made to reveal its ligand. Most studies focus on a conserved sequence in the catalytic domain, HEXGHXXGXXHS, which has significant homology with the zinc^{++}-binding region of bacterial MMPs (Reynolds, 1994). The Zn^{++}-binding capability of the first 2 histidines in this motif is now widely recognized; and recently the last histidine residue has also been proposed as Zn^{++}-ligand (Woessner, 1994). Besides the histidine residues, the glutamine residue (E) in this motif may also be important for catalytic activity. Crabbe *et al.*, (1994) have shown that gelatinase A, with replacement of this Glu by Ala, display little gelatinolytic activity (< 0.01% of wild type). The catalytic domains in gelatinase A have an additional sequence, consisting of three 58-59 amino acid repeats that share homology to gelatin-binding (type II) domains of fibronectin (Collier *et al.*, 1988). It has been suggested that these repeats are responsible for gelatinase binding with ECM components. Gelatinase B contains a collagen-like sequence and the gelatin-binding repeats.

The C-terminal domain of MMPs shows weak homology to vitronectin. Deletion of this domain may result in changes in substrate specifity (Woessner, 1994; Stetler-Stevenson *et al.*, 1993). For example, truncated collagenases (MMP-1 and MMP-8), consisting of only a catalytic domain, lose their unique ability to cleave triple helical fibrils of collagen (Woessner, 1994). The C-terminal domains of gelatinases contain extra binding sites for TIMPs.

1.3.2. **Regulation of MMP expression**

Regulation of MMP expression occurs primarily at gene transcriptional level. A variety of agents, including growth factors, cytokines, and tumor promoters (such as tetradecanoyl phorbol acetate, TPA) are involved in MMP transcriptional regulation.

The regulation of MMP expression appears to be cell specific. For example, TGF-ß can suppress MMP production in human fibroblasts; but it has no effect upon the production of MMP-1, MMP-7, and MMP-9 in human monocytes (Busiek *et al.*, 1995). The intracellular signaling events(s) induced by various agents is still not well understood. Wahl and Corcoran (1993) have proposed a PGE2-cAMP-putrescine pathway for stimulation of monocytes MMPs. McDonnell *et al.*,(1990) suggested EGF-induced stromelysin expression in rat fibroblasts involves activation of a protein kinase C pathway. In addition, a tyrosine kinase pathway and calcium influx have also been proposed to mediate extracellular signals to regulate MMP transcription (Xie *et al.*, 1994; Kohn *et al.*, 1994).

The cis-acting elements of MMP genes play an important role in transcription. In the promoter region of MMP-1, -3, -7, -9, -10, a TATA box is seen about 30 nucleotides upstream from the transcriptional start site. Further upstream, there are activator protein-1 (AP-1) and polyoma enhancer A binding factor 3 (PEA3) binding sites. Transcription factor AP-1 is

composed of dimers of protein products of c-*jun* and c-*fos* oncogenes. A variety of extracellular signals, including bFGF, EGF and phorbol esters, can upregulate production of c-JUN and c-FOS proteins, and thus influence MMP gene expression (Matrisian, 1992). The PEA3 binding sites, originally identified in polyoma virus enhancer, recognizes transcription factors of the c-ets family. Complete MMP gene induction by such factors as PDGF, bFGF and IL-1 requires the presence of both AP-1 and PEA3 binding sites. It appears that PEA3 binding sites have an added effect with AP-1. Another interesting sequence in the promoter region of rat stromelysin is a TGF-ß inhibitory element (TIE). This region is responsible for the inhibitory effect of TGF-ß on rat stromelysin gene expression. Human MMP-1, -7, and -9 gene promoters also contain TIE-like sequences. The human gelatinase A promoter, in contrast to most MMPs, has certain features of a house-keeping gene, including lack of a TATA box (Huhtala *et al.*, 1990). This promoter also lacks AP-1 binding sites or TIE elements. The unique structure of the MMP-2 gene may provide an explanation for its wide distribution in body tissues. The unique structure of the MMP-2 may also affect its response to extracellular signals: TPA, a stimulator for MMP-1 expression, inhibits MMP-2 gene expression; and TGF-ß, which inhibits MMP-1 and -3 gene expression, up-regulates MMP-2 activity. Recent evidence suggests that post-transcriptional regulation, such as the alteration of mRNA stability, also takes part in MMP gene expression (Delany and Brinckerhoff, 1992).

1.3.3. **Extracellular activation of MMPs**

All the known MMPs are secreted from cells in latent form. Activation of MMPs involves breakage of cysteine-Zn interaction and subsequent proteolytic processing. A number of agents, such as physical or biochemical agents as well as proteolytic enzymes, can initiate MMP activation

(Springman *et al.*, 1990). Among them, the role of plasmin has received most research attention (Stetler-Stevenson *et al.*, 1993). Plasmin is a serine proteinase with a variety of cleaving substrates. It has been shown that plasmin concentration and activity is largely localized around the cell surface where its precursors (plasminogen) and activators (urokinase-like plasminogen activator) both exist (Murphy and Docherty, 1992). Human MMPs, including MMP-1, -3, -7, -10, have propeptide sequences cleavable by plasmin. And it has been shown that plasmin treatment of procollagenase generates a 42 kD enzyme with proteolytic ability (Davies *et al.*, 1992). However, plasmin-treated MMPs may not be fully potentiated. Their enzymatic activity can be significantly increased in the presence of stromelysin. The same is true for trypsin- or organomercurial-treated MMP (Murphy *et al.*, 1987). Based on these observations, a cascade of proteolytic events has been proposed for the activation of ProMMPs.

The propeptide of gelatinase A lacks an apparent cleavage site to plasmin or other proteinases and its extracellular activation may be different from that of other MMPs. Emerging evidence suggests that a membrane-type matrix metalloproteinase takes part in this process (Sato *et al.*, 1994). Expression of this membrane "activator" can be induced in a number of cell types, such as fibroblasts, osteoblasts, chondrocytes, and endothelial cells, by concanavalin A, cytochalasin D, phorbol ester, TGF-ß1 or collagen (Maliti *et al.*, 1994). Strongin *et al.* (1995) have demonstrated that membrane dependent activation of MMP-2 results in an initial propeptide cleavage at Leu33. The initial cutting is followed by another cleavage at Try81 which occurs during autoproteolytic cleavage in the presence of organomercurial.

1.3.4. **Inhibition of MMP enzymatic activity and the roles of TIMPs**

Extracellularly, ECM-degrading activity of MMPs can be inhibited by certain agents, including α_2-macroglobulin and TIMPs. However, the large size of α2-macroglobulin (about 780 KD) restricts its sites of action. TIMPs are produced by many cells in the body. They bind tightly to active MMPs and function in the extracellular space of many tissues. It has been shown that TIMPs can form 1:1 stoichiometry complexes with MMPs, which block the access of MMPs to their substrates. This binding is non-covalent in nature and active inhibitors can be reversed from MMP-TIMP complexes. So far, three distinct TIMPs have been isolated and cloned. They have closely related structural and inhibitory properties (Denhardt *et al.*, 1993).

TIMP-1, a 28.5 KDa glycoprotein, is the most extensively studied TIMP. A two-domain structure has been shown for this protein: a large 3-loop, N-terminal domain possessing the metalloproteinase inhibiting activity and a small 3-loop, C-terminal domain probably important for binding to gelatinases at sites other than catalytic sites (Denhardt *et al.*, 1993). The tertiary structure of TIMP-1 is maintained by six-disulfide bonds between 12 cysteine residues of this protein. Maintenance of correct tertiary structure is essential for its inhibitory activity, as reductive agents or treatment with certain proteinases (including human elastase and trypsin) inactivate TIMP-1 (Willenbrock and Murphy, 1994). The first 22 N-terminal amino-acids following the site of signal peptide cleavage are highly conserved among TIMP-1 from many species. O'Shea *et al.* (1992) suggest that His 7 and Gly 9 in this region are active for binding with zinc at the active site of MMPs, and thus decide the inhibitory activity of TIMP-1 . TIMP-1 also contains 2 potential sites for N-glycosylation. The carbohydrate moiety may take part in binding to ECM components and thus effect retention of TIMP-1 in tissues (Stricklin, 1986).

TIMP-2 is a 21 KDa molecule that shares 40% sequence homology with TIMP-1 and lacks the sites for glycosylation. However, all the 12 cysteine residues are present in TIMP-2 and their relative position is conserved (Docherty *et al.*, 1992). Both TIMPs can form complexes with gelatinases at the COOH-terminal domain. TIMP-1 can bind with gelatinase B; and TIMP-2 binds with gelatinase A. Binding of TIMP-2 to gelatinase A at C-terminal domain prevents the auto-activation of the enzyme, and may take part in blocking further binding of enzyme to TIMPs. It has been shown that for C-terminal truncated gelatinase A, a 10-fold excess of TIMP-2 is required to give 90% inhibition of the enzymatic activity (Fridman *et al.*, 1992). A similar enzyme-inhibitor structure has been proposed for gelatinase B and TIMP-1. The most recently characterized member of the TIMP family is TIMP-3. It was originally cloned from Rous sarcoma virus transformed chicken embryo fibroblast, and its human homologue has recently been cloned and mapped to chromosome 22. The TIMP-3 has 37% and 42% peptide sequence homology to TIMP-1 and TIMP-2, respectively. It contains 12 cysteine residues at the same relative positions as other TIMPs and a potential N-linked glycosylation site near the C-terminal (Apte *et al.*, 1994). TIMPs not only inhibit MMP activity, but also function as growth promoting factors. Hayakawa *et al.* (1992) have documented that TIMP-1 promotes fibroblast and endothelial cell proliferation.

The expression of TIMP-1 and TIMP-2 is regulated primarily at gene transcriptional level. There is experimental evidence that TIMP-1 transcription is highly sensitive to stimuli, whereas TIMP-2 expression is largely constitutive (Denhardt *et al.*, 1993). Transient transfection experiments have revealed regions of cis-acting regulatory sequences in the TIMP-1 gene promoter. These sequences include AP-1 and PEA3 binding sites, which are supposed to be

important for growth factor and cytokine regulation of TIMP-1 expression (Edwards *et al.*, 1992). Post-transcriptional regulation also regulates TIMP-1 expression (Kordula *et al.*, 1992).

1.3.5. **Implication of MMPs and their inhibitors in atherosclerosis**

By studying human atherosclerotic tissues, Richardson *et al.*(1989a) suggest that proteases may take part in weakening of the fibrous cap. This is subsequently supported by Henney *et al*, (1991), who show the presence of stromelysin mRNA in atherosclerotic plaques. Further studies show the existence of all three types of MMPs in advanced atherosclerotic lesions, especially in plaque shoulder regions. More recently, Galis *et al.* (1994) have reported that these MMPs are enzymatically active. It is conceivable that MMP, by degrading ECM components, may change the focal tensile strength of atherosclerotic plaques and lead to plaque rupture, thrombosis, and hemorrhage.

Besides their roles in advanced atherosclerotic lesions, participation of MMPs in SMC migration and proliferation has also been reported (Newby *et al.*, 1994). The synthesis of MMP-1, MMP-2, and MMP-3 by SMC, especially "synthetic " SMC, has been shown (Sasaguri *et al.* 1994). And inhibitors of MMPs have been reported to inhibit proliferation and migration of cultured SMCs (Newby *et al.*, 1994; Kenagy and Clowes, 1994). Interestingly, a serine proteinase known as vascular elastase may also be involved in migration and proliferation of SMCs (Rabinovitch, 1995).

Both endothelial cells and smooth muscle cells can produce TIMPs (Libby, 1995). But the role of TIMPs in atherogenesis is still unclear. Considering the important role of ECM remodeling in atherogenesis, it is of considerable importance to investigate quantitative, as well as possible qualitative, changes of TIMPs in the process of atherosclerosis

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Hypothesis I:

Increased collagen accumulation in neointima is due to enhanced synthesis by SMC.

Chapter 2

De-endothelialization modulates collagen biosynthesis of arterial smooth muscle cells(SMCs)

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2.1. Abstract

Neointima, developed in response to aortic deendothelialization in rabbits shows an increased amount of extracellular matrix (ECM) components including proteoglycans and collagen. In this study, we examine the interaction between increased collagen accumulation and synthetic activity of neointimal SMCs using a cell culture system. Freshly confluent SMCs, derived either from normal aorta or from aortic neointima, 15 weeks after aortic injury, were used in this study. The newly synthesized collagen was measured using [^{3}H]-proline; and the mRNA expression for two major types of collagen, collagen type I and type III, was studied by Northern blot analysis. Our results showed that there is a 2-3 fold increase in the synthesis of collagenous protein by neointimal SMCs. At the same time, there is a five and a three fold increase for procollagen I mRNA and procollagen III mRNA, respectively. These data indicate that increased synthesis contributes to collagen content in the arterial neointima and both transcriptional and post-transcriptional regulation take part in this accumulation. Collagen not only takes part in platelet activation after arterial injury, but also relates to lipoprotein deposition through its high affinity with LDL.

Key Words: Collagen; ECM; metabolism; mRNA; atherosclerosis.

2.2. Introduction

Atherosclerosis is characterized by focal thickening of the walls of musculo-elastic arteries accompanied by accumulation of extracellular matrix (ECM) components and lipoproteins.[1,2] Collagen constitutes the major ECM protein in arteries. It plays an important part in atherosclerosis, not only because of its structural stabilization function in plaques, but also because of its interaction with growth factors, cytokines and lipoproteins.[3,4] As a family, collagen comprises at least 15 distinct members. Six of them are present in the vascular wall, namely types I, III, IV, V, VI and VIII collagen.[5] Both SMCs and endothelial cells synthesize collagen, even though the major role of SMCs is well established. A large amount of in vivo and in vitro data has shown that SMCs can produce all types of arterial collagen with the exception of only type VIII.[6,7]

Distribution of various types of collagen in atherosclerotic lesions has been extensively reported in the literature. Elevated collagen concentration has been shown in atherosclerotic lesions.[1,8] Among all the types of collagen, increased deposition of collagen type I and III is most predominant. Most reports indicate that these two types of collagen represent 60% of the total protein and 80-90% of the total collagenous protein in atherosclerotic lesions.[9] Further observations indicated a decreased proportion of type III to type I collagen in lesions.[10] Most of these results were obtained from biochemical analysis using aortas from autopsy. An inherent disadvantage relates to the presence of other components from arterial intima and adventitia, which can only be overcome by pure culture of SMCs.

Quantitation of various types of collagen usually employs pepsin solubilization, followed by electrophoresis or chromatography. Conflicting data generated, employing this method, reflect

its limitations.[5] Recent developments in molecular biology provide us with a powerful tool to analyze collagen synthesis. All the known types of collagen can be easily differentiated at the nucleic acid level and their amount subsequently measured.[11]

Using a nucleic acid hybridization technique, we examine the biosynthesis of collagen type I and type III by cultured SMCs from neointima, developed in response to a balloon catheter-induced deendothelialization of the rabbit aorta. Our results suggest an enhanced collagen production by neointimal SMCs; moreover the effect of de-endothelialization is more prominent on gene expression of type I collagen.

2.3. Materials and methods

2.3.1. Animals and surgical procedure

New Zealand, white, male rabbits, 2.0 - 2.5 kg body weight, were acclimatized to the animal quarters for at least two weeks. The surgical procedure to remove endothelium, identical to that reported previously,[12] was performed on a group of fifteen rabbits. Briefly, the animals were anaesthetized with pentobarbital sodium(30 mg/kg b.w.). One femoral artery was exposed and a small incision was made. Then a 4F Fogarty, arterial, embolectomy catheter (American Edwards Laboratories, Santa Ana, Calif., USA) was introduced through the incision up to the arch of the aorta. The balloon catheter was inflated with 0.75 ml saline and withdrawn with decreasing volume. The procedure was repeated three times to ensure complete deendothelialization. The catheter was then removed and the incision was closed. Another group of fifteen rabbits, used as controls, was subjected to a sham operation, i.e. the femoral artery was exposed but no catheter was inserted. Animals of both groups were maintained on normal rabbit

chow and water *ad libitum* for the rest of the experimental period.

All animal procedures carried institutational approval according to guidelines set by the Canadian Council on Animal Care.

2.3.2. Tissue preparation and smooth muscle culture

A group of 30 rabbits were randomly divided into three groups of ten animals each and euthanised at the 15th week after initial surgery. Of the ten rabbits in each group, five underwent the surgical procedure for deendothelialization and the remaining five had a sham operation. Thirty minutes before killing, the animals were injected with 5 ml of a 0.45 % solution (wt/vol) of Evans blue dye (Allied Chemical Company, New York, New York), which outlines with precision the areas where endothelium has not regenerated (deendothelialized aorta, DEA) by staining them blue, whereas the areas where endothelium has regenerated (reendothelialized aorta, REA) appeared white on a blue background. The control rabbits were also injected with Evans blue. At sacrifice, the aortas from arch to bifurcation were removed *en bloc* and then were opened longitudially. Tissues from DEA and REA were seperated; those for morphological observation were rinsed, embeded into OCT, and stored at -70°C; the tissues for cell culture were rinsed and immediately processed.

The SMC culture was performed as described previously.[12] Briefly, endothelium was removed from arterial tissues, and the upper medial layer of control aorta and the neointimal layer of the balloon-injured aortas were peeled off using Bergh cilia forceps. The tissues from control arteries, from DEA or from REA, were separately cultured. The tissue explants were first cut into 5-1.0-mm^3 pieces and the pieces were then plated in 100 mm^2 culture dishes in DMEM

containing 10% (V/V) fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. The dishes were incubated at 37°C, in 5% CO_2 and 95% air. The medium was changed twice a week. When the cells reached relative confluence in primary culture, they were harvested with 0.25% trypsin-EDTA(GIBO, Burlington, Ont., Canada) treatment and an equal number of $1X10^5$ cells were re-seeded into each culture dish. The cells from first passage were used both for Northern blot analysis and for protein analysis.

2.3.3. Light microscopic observations

The tissues from normal artery, DEA, and REA were cut with a cryostat into sections of 5-7 µm thick at -20° C, and air-dryed for 1 hr at room temperature. Then the sections were post-fixed with acetone:methanol (6:4). The tissues were examined under a microscope after staining with haematoxylin & eosin.

2.3.4. Immunofluorescence

A certain number of first passage SMCs were allowed to grow on coverslips in 35-mm petri dishes at a density of $1 X 10^5$ cells per dish. After reaching confluency, the cells were rinsed with PBS and fixed in 100% methanol for 5 min. at room temperature, then air dried for at least 1 hr. After several rinsings with PBS, the cells were incubated for 90 min. with a monoclonal, anti-α-smooth muscle actin antibody (Sigma, St Louis) at a concentration of 1:400 in PBS. As control, the cells in several dishes were incubated with non-immune rabbit serum. Fluorescent conjugated anti-mouse IgG antibody (FITC, Sigma, St Louis) was applied at a concentration of 1:32 in PBS. After 30 min. incubation with secondary antibody, the coverslips, on which the cells

grew, were rinsed several times and mounted on glass slides with 90% glycerol. The slides were observed on a fluorescence microscope (Leitz, Germany).

2.3.5. Analysis of collagenous and non-collagenous protein synthesis

We analyzed collagen synthesis with a slight modification of the method described by Okada et al.[13] The primarily cultured SMC cells were harvested and re-seeded as described above. All the measurements were started when the cells, from normal artery, DEA, and REA, had just reached a confluent state. Twenty-four hrs before analysis, the cells were washed with fresh DMEM 3 times, and then incubated with 1.0 ml DMEM containing 10% FBS, 20 μCi/ml of [H^3]-proline, supplemented with 0.1 mM sodium ascorbate and 0.5 mM ß-aminopropionitrile fumarate. Then the cells were detached with trypsin-EDTA and the cell numbers were counted. The ice-cold cell suspension was homogenized by a sonicator (Quigley-Rochester Inc. Rochester, NY). The homogenized sample was used for quantitative analysis of collagen synthesis.

2.3.6. Radioactivity counting

One aliquot of sample was boiled at 100°C for 10 min. and dialyzed thoroughly against 0.05 M acetic acid for 3 days at 4°C with gentle stirring. The sample were then collected and lyophilized in a Freeze Dryer(Lab Con Co., Canada). The lyophilized samples were redissolved in 1.0 ml 0.04 N NaOH and then neutralized with Tris-HCl buffer (pH 7.4). After digesting with collagenase (Sigma, St.Louis, MO) at 37°C for 2 hr.. The non-collagenous protein was precipitated by addition of 10% TCA and 0.5% Tannic acid. The collagenous protein in the supernant was collected. An aliquot of a 100 μl sample from collagenous fractions was mixed

with 5.0 ml of Cytoscint scintillation fluid (ICN Biomedicals, Costa Mesa,CA). The radioactivity was counted on an 1211 RACKBETA scintillation counter (LKB, Sweden). Each sample was assayed in triplicate. The results were expressed as dpm/10^5 cells.

2.3.7. RNA extraction and Northern blot analysis

Cytoplasmic RNA was extracted using TRIzol Reagent (GIBCO BRL) according to Chomcynski's method.[14] RNA thus obtained was further purified by digesting with DNase (Progema) for 60 min at 37°C. The purified RNA was separated on a 1% agarose gel containing 0.6 M formaldehyde and then transferred onto a nylon membrane (Hybond-N+, Amersham) using a semi-dry 2117 MULTIPHOR II electrophoresis unit (LKB). The membrane, with RNA immobilized by UV cross-linking, was hybridized to the respective cDNA probe. The plasmids containing cDNA of human collagen type I(α1) and type III(α1) clones were purchased from ATCC (American Type Culture Collection). The subcloning and cDNA probe preparations were performed according to the depositors' instruction. Radiolabeling of the probes was done using [α-^{32}P]-dCTP (ICN) and T7 random labeling kit (Pharmacia). Autoradiography was performed by exposing X-ray films (Kodak X-Omat AR) and intensifying screens at -70°C. The exposure time was 1 week. Autoradiograms were scanned using a Hoefer GS 3000 densitometer (LKB) to measure the mRNA signals with α-tubulin hybridization as a reference for the relative mRNA load per lane.

2.3.8. Statistical analysis

All data were expressed as mean±SD. Statistical significance was determined with one-

way ANOVA using SYSTAT software package (SYSTAT Inc., Evanston, IL). p values less than or equal to 0.05 were considered significant.

2.4. Results

Morphological observations

Figure 1 shows light micrographs of normal and denuded aorta, 15 weeks after de-endothelialization. In normal aortas, a thin, single layer of endothelium is visible (Fig. 1a). After arterial injury, however, a layer of neointimal tissue has developed on the luminal side of the aorta. Most cells in the neointima have an elongated, spindle-shaped appearance, suggesting their SMC nature. The neointimal tissue in areas covered by regenerated endothelial cells (REA) is thicker than the adjacent areas without endothelial cells (DEA, Fig. 1b and Fig. 1c).

In culture, growth of SMCs from aortic explants became visible at about 7 days in primary culture. The cells were elongated and grew radially from the explants. In the following 2 weeks, a large number of cells grew out from the explants and organized themselves in a typical "hill and valley" pattern (Fig. 2). The "hill" refers to the narrow ridges formed due to overlapping of several cell layers in some areas, whereas "valley" represents the monolayer of SMC surrounded by multilayered areas.

Figure 3 illustrates the immunofluorescence labelling of cultured cells with anti-α-smooth muscle actin antibody. The uniform and intense fluorescence reactivity indicates that these cells are arterial SMCs.

Collagen protein expression

The newly synthesized collagenous protein, labelled by [^{3}H]-proline, was measured in

SMC cultures from both normal and injured arterial tissues. All three types of SMCs synthesized measurable amounts of collagens. However, the neointimal SMCs (both from REA and DEA) synthesized more than twice the collagenous protein than did the control SMC ($p< 0.01$). The difference between B-SMC and W-SMC was not significant. Results of the measurement are graphically showed in Figure 4.

Collagen gene expression

Signals for rabbit pro-α1(I) mRNA, transcripts of 4.8 kb and 5.8 kb, were present in SMCs from both normal and injured aortas. However, the intensities of the signals were different. The SMCs from REA have the highest intensity, followed by cells from DEA. The signals in SMCs from normal aortas were the weakest among the three, but they are still clearly visible in the autoradiogram (Fig. 5A). The intensities of signals from different tissues were measured using a densitometer and normalized to the signal for mRNA of α-tubulin. As shown in Fig. 5B, the signal intensities in SMCs from DEA and REA were 5 and 6 times stronger than that from normal aorta, respectively.

Signals for pro-α1(III) mRNA were transcripts of 5.4 kb and 4.8kb. Even though this mRNA was expressed in all 3 types of SMCs, the signals in normal SMCs were weak and required a prolonged exposure of minimum two weeks to be visible on autoradiogram (Fig. 6A). Quantitative assesment showed that mRNA expression in neointimal SMCs was 3 fold higher (Fig. 6B, $p<0.05$) than in the normal SMCs. The difference between REA and DEA, however, was not significant.

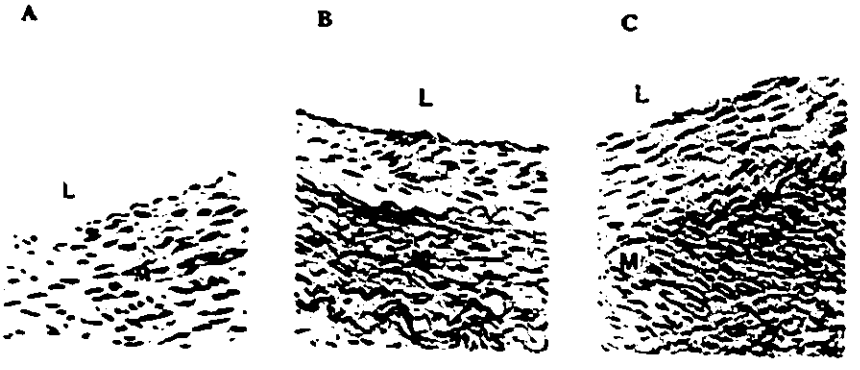

Fig. 1. Light micrographs showing intimal-medial tissues of rabbit aortas. 1A, normal aorta; 1B, neointima at DEA, without endothelial covering and appears blue by Evans blue staining; 1C, neointima at REA, the neointima is thicker and appears white by Evans blue staining. L=lumen; M=media. (H & E, original magnification X200)

Fig. 2. Micrograph of SMC in primary culture. The cells have a typical hill and valley pattern. (original magnification X100)

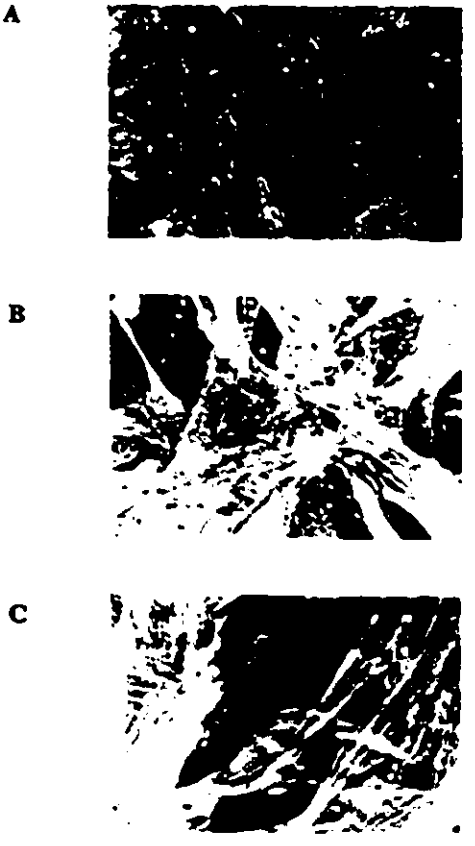

Fig. 3. Immunofluorescent micrographs of cultured SMCs. All cells have shown reaction with a specific anti-α-smooth muscle actin antibody. A, normal SMCs; B, SMCs from DEA; C, SMCs from REA. The SMCs from DEA and REA tend to grow in an overlapping pattern. (original magnification, X400)

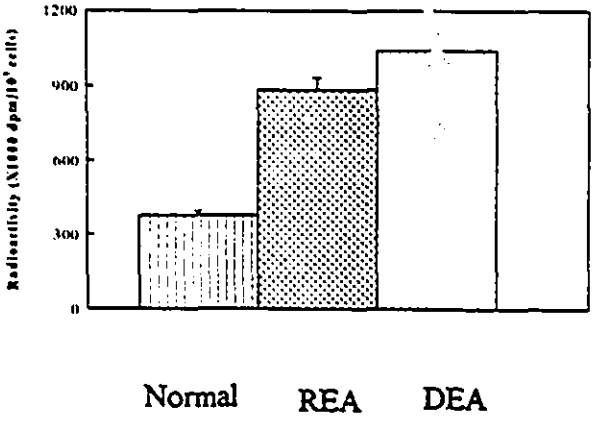

Fig. 4. [^{3}H]-proline labeled collagenous protein synthesized by SMC in culture and expressed as dpm/10^5 cells. The bars represent mean and standard deviation of incorporated radioactivity. (n=5)

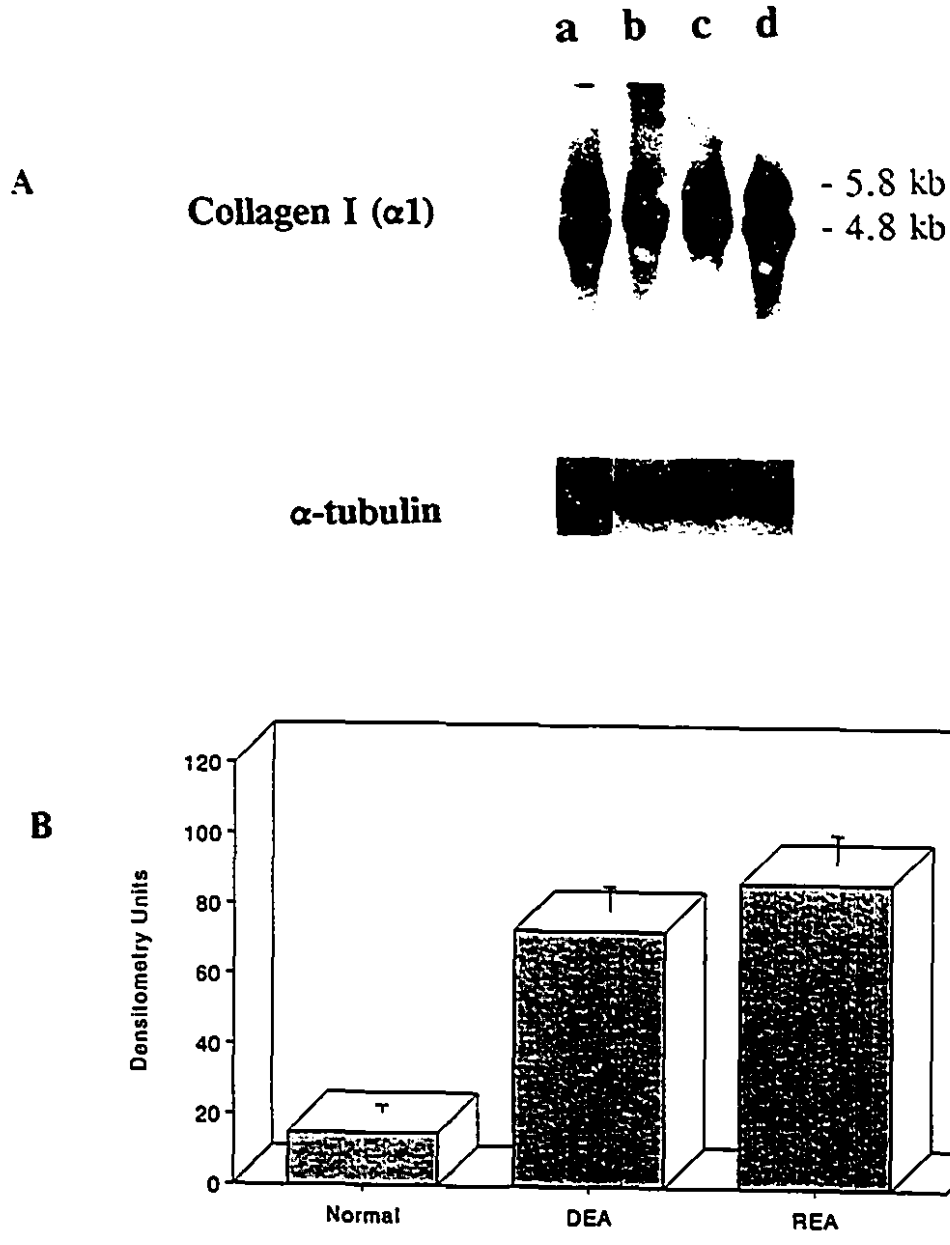

Fig. 5. Northern blot analysis of Collagen type I mRNA expression in normal and neointimal SMCs. A: Cytoplasmic RNA from human fibroblasts was used as a positive control (lane a); and RNA from normal SMCs, SMCs from DEA and from REA was loaded to lane b, c, d. The blot was densitometrically scanned and normalized to α-tubulin for quantification. B: bar graph showing signals for Collagen type I is significantly increased in neointimal SMCs ($p<0.01$). Values are represented as mean ± SD (normal SMC, 14.9 ± 1.2; SMCs from DEA, 76.0 ± 6.5; SMCs from REA, 89 ± 9.1; n=5)

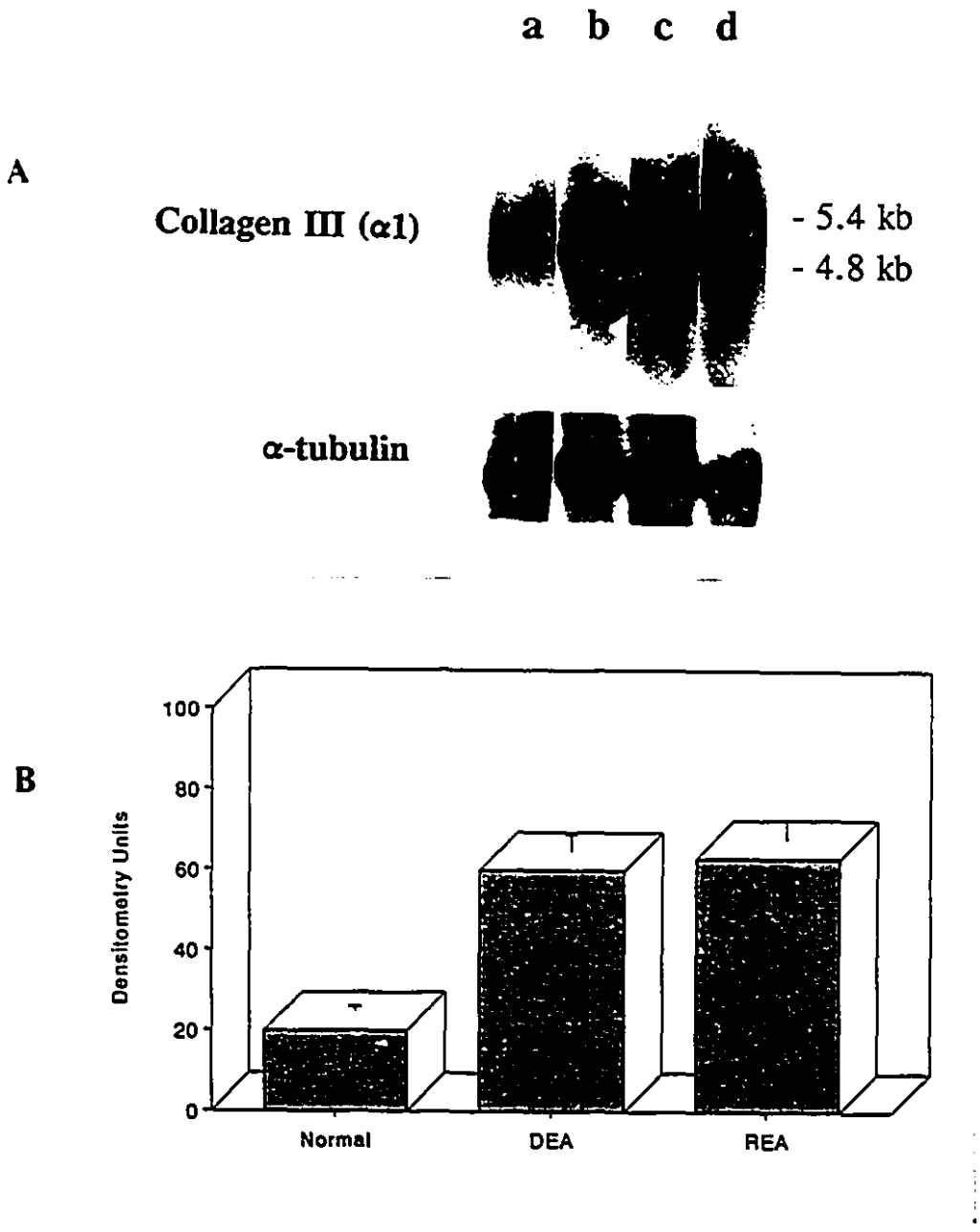

Fig. 6. Northern blot analysis of Collagen type III mRNA expression in normal and neointimal SMCs. A: Cytoplasmic RNA from human fibroblasts was used as a positive control (lane a); and RNA from normal SMCs, SMCs from DEA and from REA was loaded to lane b, c, d. The blot was densitometrically scanned and normalized to α-tubulin for quantification. B: bar graph showing signals for Collagen type I is significantly increased in neointimal SMCs ($p<0.01$). Values are represented as mean ± SD (normal SMC, 20.9 ± 2.2; SMCs from DEA, 57.7 ± 4.3; SMCs from REA, 61.4 ± 5.1; n=5)

2.5. Discussion

In this study, we showed that cultured SMC derived from injury-induced neointima synthesized twice the amount of collagen protein than cells derived from normal artery. In addition, mechanical injury altered the gene expression ratio of collagen type I and type III. De-endothelialization caused a 5-6 fold increase in procollagen I(α1) mRNA and about a 3 fold increase in procollagen III(α1) mRNA. These data indicated that the collagen type I to type III ratio is increased in the neointima, a lesion similar to atherosclerotic plaque in man.

Balloon catheter de-endothelialization is widely employed in inducing experimental atherosclerosis. This injury has been reported to initiate a complex cascade of interaction and events resembling those seen in atherosclerosis.[15] In rabbits, we and others have shown this manipulation leads to SMC migration, proliferation, ECM accumulation and thus neointimal formation; in long term experiments, we have also shown deposition of lipoprotein in the neointimal tissue of normally fed rabbits.[16] Numerous reports have documented the similarities between neointimal and atherosclerotic SMCs. For example, both cell types are rich in endoplasmic reticulum, Golgi apparatus and ribosome; at the same time, both of them have decreased myofilment volume compared with their cells of origin. Probably more importantly, both cell types have enhanced synthetic ability and produce more ECM components.[17] A major concern about neointimal SMCs is the duration of their phenotypic change. Results from this laboratory indicate that SMC phenotypic change might be a persistent process. For example, a recent study from this laboratory has shown that enhanced ability to synthesize proteoglycan of neointimal SMCs extended to 15th weeks after de-endothelialization.[18] Our results of enhanced

collagen synthesis 15 weeks after endothelial injury give further support to the persistence of the phenotypic alteration of SMCs.

Many studies of collagen production in aortic tissues or cells have been performed by measuring the metabolic incorporation of radiolabelled proline into hydroxyproline. The latter is employed as an index of collagen synthesis.[13] This method is effective in measuring newly synthesized collagen by various tissue and organ cultures, but it is not able to identify the change of any particular type of collagen. Two approaches are commonly applied in characterization of various types of arterial collagen. The first involves immunohistochemical staining using type-specific anti-collagen antibodies.[10] Obviously, this method does not allow quantitative evaluation of collagen. The second approach utilizes pepsin solubilization of ECM followed by sodium dodecyl sulfate (SDS) gel electrophoresis, or chromatography. However, the extent of pepsin proteolysis is hard to control and data derived employing this method are conflicting. In contrast to the complex biochemical analysis, all types of collagens can be easily separated at nucleotide level. In fact, certain types of collagen were first identified at the molecular level.[19] So, the nucleotide hybridization technique, such as Northern blot analysis, provides a powerful quantitative tool for analysis of various types of collagen.[20]

The increased collagen synthesis observed in this study may relate to altered production of growth factors and cytokines and their receptors. These mitogens can up-regulate collagen production at both the transcriptional and post-transcriptional levels.[21] Transforming growth factor-ß1 and platelet derived growth factor (PDGF) are two important regulators implicated in the development of atherosclerosis. Schlumberger et al[22] reported the enhancement of production of collagen and noncollagen proteins by the co-culture of arterial SMCs and TGF-ß1; moreover

the increased rate of net collagen production was twice as high as that of noncollagenous proteins. PDGF is composed of two related polypeptides, designated as A and B chains. Amento[23] et al showed that PDGF could stimulate collagen synthesis in cultured SMCs. The stimulatory effect of PDGF is largely mediated by its proliferation-promoting function. Employing a solid-phase ELISA method, we have recently shown that de-endothelialization stimulates the SMC production of TGF-ß1 three fold and PDGF-AB fifteen fold.[24] In another study, we also showed that deendothelialization upregulates the mRNA expression of the PDGF-ß receptor about 2 fold.[25] Besides SMCs, macrophages and lymphocytes in the atherosclerotic plaque can produce various growth factors and cytokines, which may also contribute to collagen synthesis.[26,27]

As the major ECM protein in atherosclerotic plaque, collagen contributes significantly to the occlusive nature of atherosclerosis. Besides, collagen facilitates lipoprotein retention in the lesions. It has been shown that type I and type III collagen have high affinity for LDL, especially for oxidized LDL.[28] Co-localization of fibrillar collagen and lipid-vesicles in both early and late atherosclerosis has been observed.[29] In our model, de-endothelialization also causes direct contact of collagen with platelets. This contact activates platelets and causes the secretion of factors like PDGF, TGF-α, TGF-ß and TxA_2, which stimulate SMC proliferation and accelerate atherogenesis.[30]

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Hypothesis II:

Neointimal ECM is functionally related to SMC migration and proliferation.

CHAPTER 3

Expression of syndecan-1 by rabbit neointimal smooth muscle cells (SMCs)

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3.1. Abstract

Enrichment of extracellular matrix (ECM) components is prominent in early atherogenesis, contributing to SMC migration and proliferation, a hallmark of the atherosclerotic lesion. A family of integral membrane proteoglycans termed syndecans has been recently recognized as a cell surface receptor for various ECM components. The syndecans are also capable of binding to growth factors. Among syndecans, syndecan-1, the first isolated member, has received most research attention. In this study, we examined the expression of syndecan-1 in rabbit aorta and aortic neointima, developed in response to a balloon catheter-induced deendothelialization. The tissues were processed for Northern blot analysis, in situ hybridization, and immunohistochemical staining. Our results indicate that in normal aorta, the signal for syndecan-1 is weak. However, arterial injury induces syndecan-1 expression at both mRNA and protein levels. The presence of syndecan-1 in the neointimal tissue is persistent, prominent even at the 12th week after injury. Syndecan positive cells are distributed in the whole layer of the neointima, but are not visible in the underlying media. The presence of syndecan-1 in arterial neointima suggests a novel means of mediating interactions between neointimal cells and their stimlating agents, including various ECM components and growth factors.

Key Words: Syndecan-1; neointima; ECM; atherosclerosis; in situ hybridization

3.2. Introduction

Migration and proliferation of smooth muscle cells (SMCs) are prominent features of atherosclerosis.[1] It has been well established that SMCs are the principal cellular component of human atherosclerotic plaques.[2] The accumulated SMCs take part in lipoprotein retention and contribute to the occlusive nature of atherosclerosis.[3,4] Activation of arterial SMC is largely mediated by ECM components as well as growth factors and cytokines.[5,6]

There are reports indicating the importance of ECM components in regulation of cell shape, adhesion, migration and proliferation.[7] The cell-ECM interaction is mediated by cell surface molecules. One family of these molecules is called integrins[8] and recent studies reveal another family of such proteins, heparan sulfate proteoglycan (HSPG). Much of the information about HSPG-cell interaction is derived from the study of a group of transmembrane HSPG known as syndecans.[9]

Four distinct types of syndecan have been identified and termed syndecan-1, -2, -3 and -4, respectively. All syndecans have a similar protein domain structure and gene exon organization. Their amino acid sequences in transmembrane as well as cytoplasmic domains are also highly conserved. However, the extracellular domains of different syndecan types varies greatly.[10] Tissue expression and biological functions of different syndecans are the focus of current research in many laboratories.[11,12] Syndecan-1 is a hybrid PG with both heparan sulfate and chondroitin

sulfate side chains. Its expression in tissues is highly regulated.[9] For example, syndecan-1 is expressed in condensing mesenchyme of lung, kidney and limb; but it is non-detectable in terminally differentiated sketetal or cardiac muscles.[13] Growth factor treatment can also increase syndecan-1 expression in certain mesenchymal cells.[14] The extracellular domain of syndecan-1 binds to bFGF and many ECM proteins, including fibrillar collagens (collagen type I, III, V), fibronectin, thrombospondin, and tenascin.[12,15] The intracellular domain of syndecan-1 is associated with actin of the cytoskeletal system.[16] It has been suggested that syndecan-1 acts as a receptor for ECM components.

ECM, a compositional element of arterial wall, is enriched in atherosclerotic lesions.[4] Stimulated SMCs and endothelial cells are capable of synthesizing a variety of ECM components, including collagen, proteoglycan and fibronectin.[17,18] These ECM components, on the other hand, reciprocally affect cellular function. For example, heparan sulfate derived from confluent endothelial cells and SMCs can inhibit SMC proliferation.[19] And hyaluronan as well as fibronectin facilitate cellular migration.[20,21] It has been suggested that hayluronan induces migration of arterial SMCs through its interaction with a receptor for hyaluronan-mediated mobility.[22] However, mechanisms of cell-ECM interaction are still far from clear.

In an attempt to explore the role of syndecans in mediating SMC-ECM interaction, we studied the expression of syndecan-1 in rabbit neointimal SMCs, developed in response to a balloon catheter induced de-endothelialization. Our results indicate that neointimal SMCs express syndecan-1 at both mRNA and protein level, and the tissue distribution suggests a role in SMC migration and proliferation.

3.3. Materials and methods

3.3.1. Animals and surgical procedure

New Zealand, white, male rabbits, 2.0-2.5 kg body weight, were acclimatized to the animal quarters for at least two weeks. The surgical procedure to remove endothelium, identical to that reported previously,[17] was performed on a group of ten rabbits. Briefly, the animals were anaesthetized with pentobarbital (30 mg/kg b.wt.). One femoral artery was exposed and a small incision was made. Then a 4F Fogarty, arterial, embolectomy catheter (American Edwards Laboratories, Santa Ana, Calif., USA) was introduced through the incision up to the arch of aorta. The balloon catheter was inflated with 0.75 ml saline and withdrawn with decreasing pressure. The procedure was repeated three times to ensure complete deendothelialization. The catheter was then removed and the incision was closed. Another group of ten rabbits, used as controls, were subjected to a sham operation, i.e. the femoral artery was exposed but no catheter was inserted. Animals of both groups were maintained on normal rabbit chow and water *ad libitum* for the ensuing 12 weeks.

All animal procedures carried institutional approval according to guidelines set by the Canadian Council on Animal Care.

3.3.2. Tissue preparation

The rabbits were killed at 4th, 8th and 12th weeks after de-endothelialization. Thirty minutes before killing, the animals were injected with 5 ml of a 0.45 % solution (wt/vol) of Evans blue dye (Allied Chemical Company, New York, New York), which outlines with precision the

areas where endothelium has not regenerated (deendothelialized area, DEA) by staining them blue, whereas the areas where endothelium has regenerated (reendothelialized area, REA) appeared white on a blue background. At sacrifice, the aortas from arch to bifurcation were removed *en bloc* and then were opened longitudinally. For in situ hybridization, tissues from normal or reendothelialized areas were selected and fixed immediately. For immuno-histological study, tissues from normal and injured areas were selected and frozen in -80°C isopentine. For RNA extraction, the endothelium was removed from the aorta and the intima-media layer was peeled from the adventitia using Bergh cilia forceps as described elsewhere[17].

3.3.3. RNA extraction and analysis

Cytoplasmic RNA was extracted using TRIzol Reagent (GIBCO BRL) according to Chomcynski's method.[23] RNA thus obtained was further purified by digesting with DNase (Progema) for 60 min at 37°C. The purified RNA was used for Northern blot analysis.

Syndecan-1 mRNA expression was studied using Northern blot analysis. Cytoplasmic RNA was separated on 1% agarose gel containing 0.6 M formaldehyde and then transferred onto a nylon membrane (Hybond-N+, Amersham) using a semi-dry 2117 MULTIPHOR II electrophoresis unit (LKB). The membrane, with immobilized RNA (by UV cross-linking), was hybridized to a syndecan-1 cDNA probe. This probe was prepared from a plasmid containing rat syndecan-1 cDNA (A gift from Dr. D.J. Carey, Sigfried and Janet Weis Center for Research, Geisinger Clinic, Danville, Pennsylvania). Radiolabeling of this probe was done using [α-^{32}P]-dCTP (ICN) and T7 random labeling kit (Pharmacia). Autoradiography was performed by exposing X-ray film (Kodak X-Omat AR) and intensifying screens for 2 weeks at -70°C.

Autoradiograms were scanned using a Hoefer GS 3000 densitometer (LKB) to measure the TIMP-1 signal with α-tubulin hybridization as a reference for relative mRNA load per lane.

3.3.4. In situ hybridization analysis

a. Preparation of probe

A plasmid vector containing rabbit syndecan-1 (about 300 bp) was prepared as described above. The plasmid was linearized with restriction enzymes. RNA probes were generated either with T7 or with SP6 polymerase (Amersham) and radiolabeled with [α-^{35}S]-UTP. The specific activity of these probes was usually 1.5X10^{6} cpm/ng RNA. Both sense and anti-sense RNA probes were produced. The anti-sense probe hybridized with mRNA in tissue and the sense probe served as a negative control.

b. Hybridization procedure

Tissues for in situ hybridization were fixed in 4% paraformaldehyde containing sucrose, frozen in liquid nitrogen and cut into 5 µm-thick sections using a cryostat. The sections were then placed on 0.1% polylysin (sigma)-coated slides. Prior to hybridization, the specimens were hydrated in PBS, incubated with Triton X-100 (0.3% in PBS), treated with proteinase K (1 µg/ml Tris-EDTA buffer) and post-fixed using 4% paraformaldehyde. To reduce non-specific binding, the specimens were treated with 10 mM iodoacetamide and 10 mM N-ethyleneimide. The specimens were pre-hybridized for 2h at 42°C in 2 X SSC solution containing 50% formamide. Twenty microliters of hybridization mixture was applied to every section. The 20 µl hybridization mixture contained 18 µl hybridization buffer (50% deionized formamide, 5 X Denhardt's solution,

10% dextran sulfate, 0.5% sodium pyrophosphate, 0.5% SDS and 250 µg/ml denatured salmon sperm DNA) and 2 µl probe solution(radioactivity more than $0.8X10^6$ cpm). The hybridization was carried out in a moist chamber for 16h at 42°C. To remove unhybridized RNA, sections were washed in RNase-containing (20 µg/ml) 2 X SSC solution for 30 min at 42°C. Posthybridization washing was performed for about 2h at 40°C in a 0.1 X SSC solution with several changes of fresh solution in between. After washing, the specimens were dehydrated in 70 %, 90% and 100% ethanol containing 0.3M ammonium acetate and dried. The sections were then immersed in an emulsion (LM-1 Amersham) and exposed for 10 days at 4°C in the dark. Finally, the autoradiograms were developed, fixed and counter-stained by hematoxylin for further examination.

3.3.5. Immunohistochemical staining

Six micron cryostat sections were placed on poly-L-lysin (Sigma, St Louis, MO) coated slides. The slides were air-dried for 1 hr and post-fixed with acetone-methanol (60-40% V/V) for 7 min. Then, the slides were air-dried for another 1 hr. The sections were incubated overnight at 4 C with monoclonal antibody to mouse syndecan-1 (a gift from Dr. M. Bernfield, Harvard Medical School, Boston, MA), diluted (1:100) in Tris-HCl buffer solution (TBS, 0.05M, pH=7.6). The slides were then rinsed with TBS followed by incubation for 30 min at room temperature with rabbit anti-rat secondary antibody (DAKO, Z-147, Carpinteria, CA), diluted (1:30) in 20% rabbit serum. Following washing in TBS, sections were incubated with rat APAAP (DAKO, D488, Carpinteria, CA), diluted in rabbit serum. The sections were then extensively

washed in TBS, developed with Fast Red and counterstained with Haematoxylin.

3.3.6. Statistics

The statistical analysis was performed by one-way ANOVA, employing the SYSTAT software package (Systat Inc. Evanston, IL). The p values less than or equal to 0.05 were considered significant.

3.4. Results

Northern Blot analysis

After hybridization with the radiolabelled syndecan-1 cDNA probe, no band was visible in the lane of cytoplasmic RNA from normal aortic SMCs, even after 2 weeks exposure. However, syndecan-1 probe did hybridize to cytoplasmic RNA from aortic neointima, and produced a predominant band of 2.6 kb and a minor band of 3.2 kb. As shown in figure 1, signals for syndecan-1 mRNA were intense in neointimal SMCs at the 4th and 8th week after initial injury; the signal was decreased, but still clear, at the 12th week after injury. Indeed, the intensity of syndecan-1/α-tubulin mRNA signal in the 12th week was only 1/3 of that in the 4th and 8th week ($p<0.01$, Fig. 2).

In situ hybridization

In order to investigate the spatial localization and to identify the cellular source(s) of syndecan-1 mRNA in vivo, we utilized in situ mRNA hybridization technique. The neointima,

developed in response to injury, exhibited a clear signal for syndecan-1. The signal is strong in neointimal sections taken at the 4th and 8th week after arterial injury. The syndecan-1 positive cells were homogenously distributed over the whole layer of the neointima (Fig. 3 a and 3b). The majority of the neointimal cells were SMCs as indicated by immunostaining with SMC specific α-actin (data not shown). No positive signal for syndecan-1 was seen in the medial layer of either normal or injured aorta. The sections incubated with the sense probe did not give any signal for syndecan-1 (Fig. 3c).

Immunohistochemical staining

The translation of syndecan-1 mRNA in the aortic neointima was verified by immunostaining with monoclonal anti-syndecan antibody. As shown in figure 4a and 4b, staining was strong in the neointimal tissues taken at the 4th and 8th week after initial injury. The staining became weaker in the neointimal tissues taken at the 12th week after injury (not shown). Again, the positive cells were restricted to the neointimal layer. Control staining, ie, staining without primary antibody, did not show any positive cells in tissue sections (Fig. 4c).

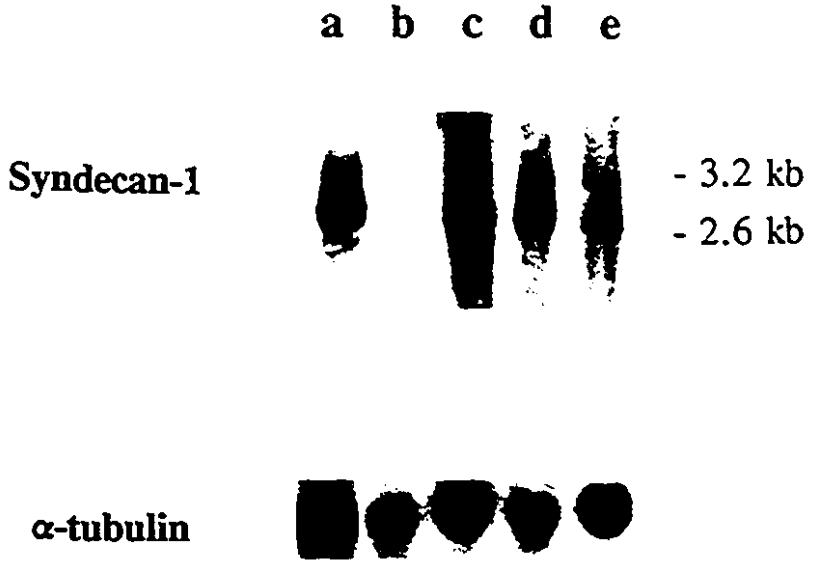


Fig. 1. Northern blot analysis of syndecan-1 mRNA expression. a: positive control using RNA from rabbit mammary gland; b: RNA from normal aortic media; c: RNA from neointima developed 4 weeks after injury; d: RNA from neointima developed 8 weeks after injury; e: RNA from neointima developed 12 weeks after injury. The blot was densitometrically scanned and normalized to α-tubulin for quantification.

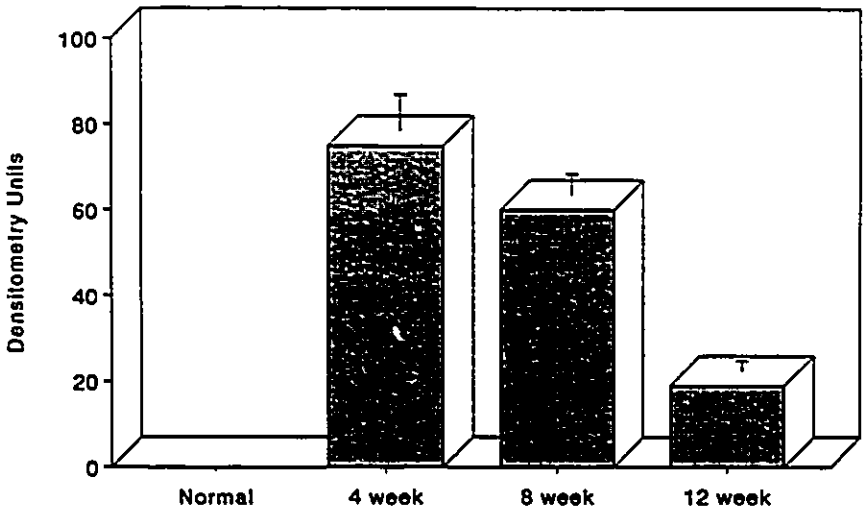


Fig. 2. Bar graph showing measurement of signal for Syndecan-1. It is not measurable in normal aortic media; however, a great increase in neointimal tissues is observed. Values are represented as mean ± SD (neointima 4 weeks after injury, 78.0 ± 8.5; neointima 8 weeks after injury, 59.5 ± 6.0; neointima 12 weeks after injury, 16 ± 2.0; n=5). The difference between 4th week and 12th week and that between 8th week and 12th week are significant ($p<0.01$).

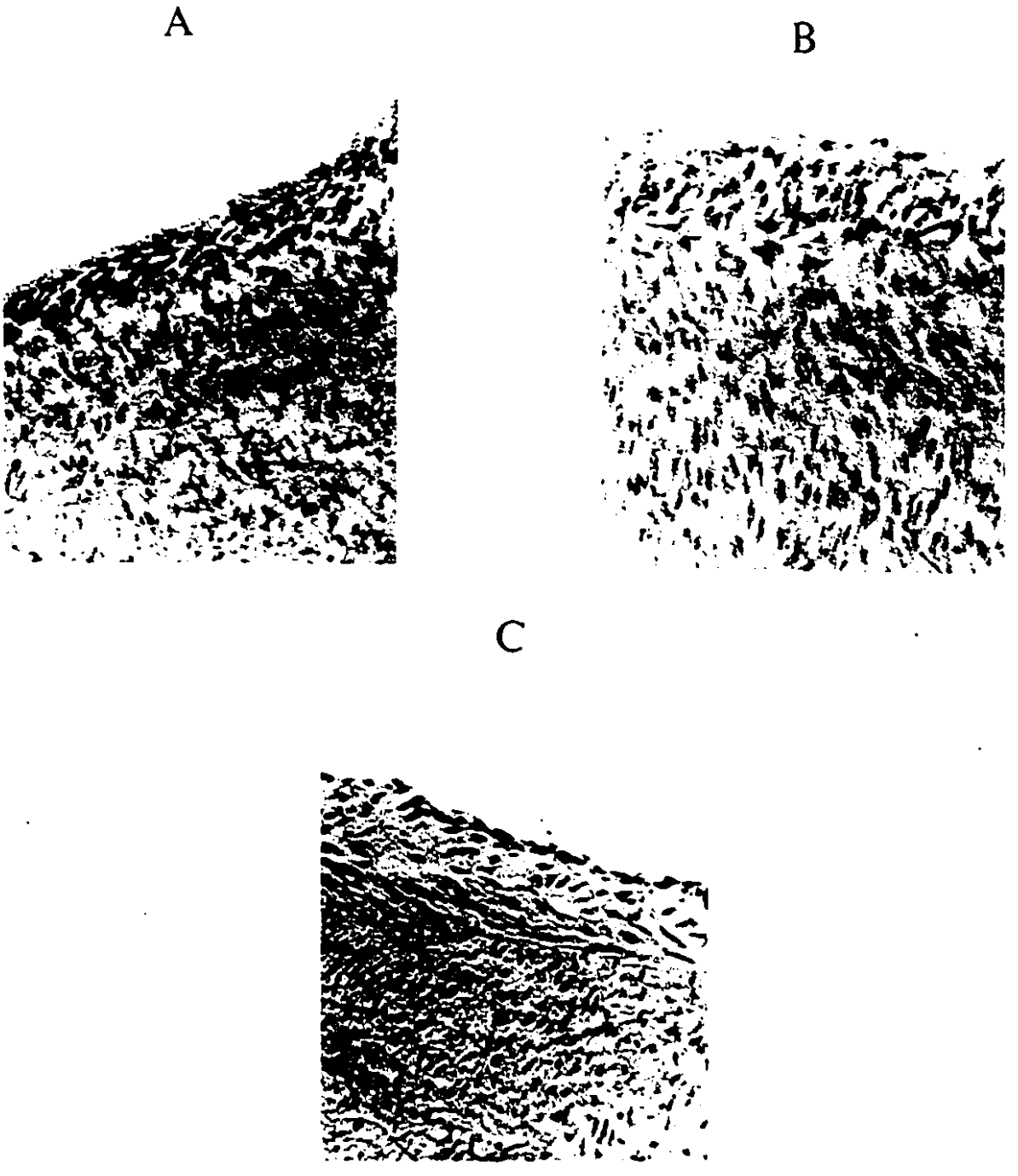
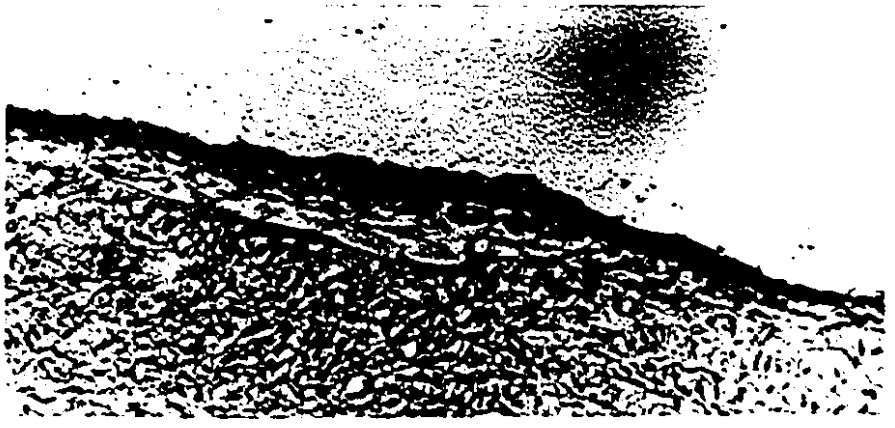


Fig. 3. In situ hybridization of deendothelialized rabbit aortic tissues with ^{35}S-labeled RNA probe for Syndecan-1, counterstained with hematoxylin. A: neointima developed 4 weeks after injury; B: neointima developed 8 weeks after injury; C: negative control using sense probe to Syndecan. Original magnification X200.

A

B



C

Fig. 4. Immunohistochemical staining with monoclonal antibody to Syndecan-1. A: neointima develpoed 4 weeks after injury; B: neointima developed 8 weeks after injury; C: negative control, staining without primary antibody.

3.5. Discussion

The SMCs in adult arteries are highly differentiated cells, which function to maintain arterial tone.[24] Injury to endothelial cells causes phenotypic alteration of SMCs with expression of genes apparent in fetal life.[25] In this study, we showed that balloon catheter de-endothelialization induced syndecan-1 expression by SMCs at both mRNA and protein level. The signal for syndecan-1 is persistent, prominent even at the 12th week after initial injury. The in situ hybridization study indicates that syndecan positive cells are visible over the whole layer of neointima.

Neointimal SMCs share several features with fetal arterial SMCs: both types of cells synthesize large amounts of ECM; and in culture, they show epithelioid morphology, proliferate without platelet-derived growth factors, and secrete PDGF.[25] It has been suggested that neointimal SMCs are de-differentiated cells, regaining characteristics of fetal SMCs.[26] The expression of syndecan-1 in the neointima shown in this study provides further evidence for this suggestion. In mature individuals, syndecan-1 is predominantly localized to epithelium.[9] This may explain the lack of syndecan-1 in adult aortic SMCs. On the other hand, expression of syndecan-1 in certain embryonic mesenchymal tissues is well documented.[12] In fact, cDNA for rat syndecan-1 core protein has been cloned from a neonatal rat aortic cDNA library.[11] It is possible that by de-differentiation, neointimal SMCs resume the capability of syndecan-1 expression. Growth factors and cytokines may also take part in syndecan expression by SMCs. One recent report indicates that TGF-ß treatment up-regulates syndecan-1 expression in NIH-3T3 cells (a fibroblast cell line).[14] This cytokine may be implicated in the observed syndecan expression as an

increased concentration of TGF-ß in rabbit neointima has been reported.[27]

Syndecan-1, through its binding to ECM components and growth factors, may influence proliferation and migration of neointimal SMCs. Among the ligand for syndecan-1 is an extracellular glycoprotein termed thrombospondin (TSP). TSP is a secreted product of many cell types, including platelets, SMCs and endothelial cells.[28] Moreover, its synthesis and secretion in SMCs are stimulated by PDGF and heparin.[29] Majack et al.,[30] have shown that TSP enhances proliferation of vascular SMCs, an effect potentiated by simultaneous addition of epidermal growth factor (EGF). In addition, monoclonal antibody to TSP inhibits SMC mitogenesis and reduces the association between cells and TSP.[31] Syndecan-1 may also regulate SMC proliferation through its interaction with serum growth factors. Numa et al.,[32] have shown that over-expression of syndecan-1 in a renal epithelial cell line results in a higher cellular growth rate in serum-rich media; however, in serum-deprived medium this over expression is associated with a proliferative disadvantage. Kiefer et al.,[33] have shown that syndecan-1 can bind to bFGF via its heparan sulfate chains. Considering the large number of heparin binding growth factors (including acidic FGF and EGF), it is also possible that syndecan-1 binds growth factors other than bFGF. Binding with syndecan-1 will influence the activity of growth factors. It has been suggested[34] that syndecan-1 or a similar cell surface HSPG is necessary for stimulating the tyrosine kinase bFGF receptor. Recent reports also suggest that binding with HSPG (including syndecan-1) will protect growth factors from inactivation.[35]

The neointimal syndecan-1 may have dual roles for cellular migration. On the one hand, it may bind to fibronectin and facilitate cellular migration. For example, cultured mesenchymal cells move across a filter to the side richest in fibronectin.[36] And fibronectin is enriched in the

migratory pathways for embryonic cells, such as that for neural crest cells.[37] Fibronectin has also been implicated in migration of arterial SMCs. In the ductus arteriosus, fibronectin around SMCs is reponsible for migration of the cells to arterial intima.[22] On the other hand, tight binding of this HSPG to certain ECM components may cause cells (such as epithelial cell) to be less mobile.[37] In fact, these functions may not be mutually exclusive. It is believed that both translocation of cells on substrate and establishment of cell-ECM adhesion are essential processes for cell migration.

In summary, we showed that the expression of syndecan-1 in rabbit aortic neointima, developed in response to a balloon catheter deendothelialization. This expression is persistent, lasting at least for 12 weeks after the injury. Syndecan-1 may act as a cell surface receptor for ECM components and contribute to the proliferation and migration of SMCs.

3.6. Acknowledgements

We wish to express our thankfulness to Dr. D.J. Carey (Sigfried and Janet Weis Center for Research, Geisinger Clinic, Danville, Pennsylvania) for the generous gift of plasmids containing rat syndecan-1 cDNA; and to Dr. M. Bernfield (Harvard Medical School, Boston, MA) for the gift of monoclonal antibody to mouse syndecan-1. We are also grateful to Dr. Serge Jothy and Dr. Qutayba Hamid of McGill University for the use of the molecular biology laboratory facility in the Department of Pathology. This work was supported by the Medical Research Council of Canada grant (#MT10590).

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Hypothesis III:

MMPs take part in ECM degradation which is a pre-requisite for SMC migration.

Chapter 4

Expression of mRNA for metalloproteinases and their inhibitor by rabbit neointimal smooth muscle cells

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4.1. Abstract

Remodelling of arterial extracellular matrix (ECM) in atherogenesis refers not only to increased deposition of proteins like collagen and proteoglycans, but also involves enhanced ECM catabolism. A family of zinc-containing proteinases, termed matrix metalloproteinases (MMPs), have recently been implicated in atherosclerosis. The activity of these MMPs is regulated by their endogenous inhibitors, called tissue inhibitors of metalloproteinases (TIMPs). In this study, we examined the expression of mRNA for gelatinases (MMP-2, MMP-9) and TIMP-1 in neointima, developed in response to a balloon catheter-induced deendothelialization of rabbit aorta. Results showed that expression of mRNA for MMP-2 and MMP-9 is increased in neointimal SMCs. This increase continued up to 8 weeks for MMP-2 and up to 12 weeks for MMP-9. In situ hybridization showed that MMP positive cells were uniformly distributed in arterial neointima and were visible in the arterial media. Expression of TIMP-1 mRNA in neointima was also increased and TIMP-1 positive cells, primarily SMCs, were localized to the neointima. The data suggest that synthesis of MMPs and TIMP-1 is a relatively prolonged process and arterial SMCs are a major source of MMP production. Enhanced synthesis of MMPs relative to that of TIMPs may contribute to migration and proliferation of SMCs, and thus to the process of atherogenesis.

Key Words: MMP; TIMP; SMC; ECM; Atherosclerosis;

4.2. Introduction

Remodeling of arterial extracellular matrix (ECM) occurs in atherogenesis.[1,2] Until recently, remodeling predominantly referred to an abundance of some of the ECM components and their redistribution. Many studies have shown that enrichment of ECM components like collagen and proteoglycans is not only critical to the initiation of the process but is also a characteristic feature of atheroma.[3] Many ECM components could induce cellular proliferation through their interaction with lipoprotein and growth factors thereby facilitating the development of atheroma.[4,5] Recently some reports, however, placed emphasis on enhanced catabolism of the ECM in advanced atherosclerotic lesions.[6,7] Accelerated ECM catabolism preceding plaque rupture has underscored the importance of ECM degradative processes in atherosclerosis.[8,9]

ECM catabolism is attributed to a family of zinc containing proteinases, called *matrix metalloproteinases* (MMPs). The MMPs have been divided into three subclasses: collagenases, gelatinases and stromelysins according to their substrate specificity. They are calcium dependent and highly regulated enzymes.[10] The MMPs are generally secreted by the cells in an inactive state known as pro-MMP. A cleavage of a specific peptide sequence of pro-MMPs by other proteases converts them to MMPs. It is generally believed that molecular transcription regulates the amount of pro-MMP to be secreted by the cells. The MMPs can be effectively inactivated by endogenous inhibitors, known as *tissue inhibitors of metalloproteinase* (TIMP). These TIMPs have been further

classified according to their peptide sequences as TIMP-1, TIMP-2, and TIMP-3.[11,12] Alteration at any regulation level may affect MMP activity. For example, enhanced MMP activity due to excessive gene transcription is implicated in metastasis of tumor cells. The tissue injury in osteoarthritis has also been attributed to MMP and TIMP synthesis.[13,14] More recently, reports have described the MMP activity in advanced atherosclerotic lesions.[5-7,15] Few studies have addressed alteration of MMPs and their inhibitors simultaneously in the early development of atherosclerosis.[16]

We examined the expression of mRNA for gelatinases (MMP-2, MMP-9) and TIMP-1 in neointima, developed in response to a balloon catheter-induced deendothelialization of rabbit aorta. Results indicated an enhanced synthesis of MMPs relative to the synthesis of TIMPs by neointimal SMCs, highlighting the importance of MMPs in atherogenesis.

4.3. Materials and methods

4.3.1. Animals and Surgical Procedure

New Zealand, white, male rabbits, 2.0 - 2.5 kg body weight, were acclimatized to the animal quarters for at least two weeks. The surgical procedure to remove endothelium, identical to that reported previously,[17] was performed on a group of fifteen rabbits. Briefly, the animals were anaesthetized with sodium pentobarbital (30 mg/kg b.w.). One femoral artery was exposed and a small incision was made. Then a 4F Fogarty, arterial, embolectomy catheter (American Edwards Laboratories, Santa Ana, Calif., USA) was introduced through the incision up to the arch of the aorta. The balloon catheter was inflated with 0.75 ml saline and withdrawn with decreasing pressure. The procedure was repeated three times to ensure complete deendothelialization. The catheter was then removed and the incision was closed. Another group of fifteen rabbits, used as controls, were subjected to a sham operation, i.e. the femoral artery was exposed but no catheter was inserted. Animals of both groups were maintained on normal rabbit chow and water *ad libitum* for the rest of the experimental period.

All animal procedures carried institutional approval according to guidelines set by the Canadian Council on Animal Care.

4.3.2. Tissue Preparation

The rabbits were randomly divided into three groups of ten animals (5 injured and 5 sham operated) each and were euthanised at the 4th, 8th, and 12th week after balloon catheter

deendothelialization. Thirty minutes before killing, the animals were injected with 5 ml of a 0.45 % solution (wt/vol) of Evans blue dye (Allied Chemical Company, New York, New York), which outlines with precision the areas where endothelium has not regenerated (deendothelialized aorta, DEA) by staining them blue, whereas the areas where endothelium has regenerated (reendothelialized aorta, REA) appeared white on a blue background. The control rabbits were not injected with Evans blue. At sacrifice, the aortas from arch to bifurcation were removed *en bloc* and then were opened longitudially. For in situ hybridization, tissues from normal aorta or from REA were selected and fixed immediately. For RNA extraction, endothelium was removed from the aorta and the intima-media layer was peeled from the adventitia using Bergh cilia forceps as described elsewhere.[18]

4.3.3. RNA Extraction and Northern Blot Analysis

Cytoplasmic RNA was extracted using TRIzol Reagent (GIBCO BRL) according to Chomcynski's method.[19] RNA thus obtained was further purified by digesting with DNase (Progema) for 60 min at 37°C. The purified RNA was separated on 1% agarose gel containing 0.6 M formaldehyde and then transferred onto a nylon membrane (Hybond-N+, Amersham) using a semi-dry 2117 MULTIPHOR II electrophoresis unit (LKB). The membrane, with immobilized RNA (by UV cross-linking), was hybridized to its respective cDNA probe. The probe for rabbit TIMP-1 was prepared as described in a previous study;[17] the probes for rabbit 92 kDa gelatinase and human 72 kDa gelatinase were prepared from plasmids kindly provided by Dr. M. Elizabeth Fini (Department of Ophthalmology, New England Medical Center, Boston, MA). Radiolabeling of the probes was done using [α-^{32}P]-dCTP (ICN) and T7 random labeling kit (Pharmacia). Autoradiography was performed by exposing X-ray film (Kodak X-Omat AR) with intensifying screens for 1 week at -

70°C. Autoradiograms were scanned using a Hoefer GS 3000 densitometer (LKB) to measure the mRNA signals with α-tubulin hybridization as a reference for relative mRNA load per lane.

4.3.4. In situ Hybridization Analysis

Plasmid vectors containing cDNA probes were obtained as described earlier. The plasmids were linearized with restriction enzymes. RNA probes were generated with either T7 or SP6 polymerases (Amersham) and radiolabeled with [α-^{35}S]-UTP. The specific activity of these probes was usually 1.5 X 10^6cpm/ng RNA. Both sense and anti-sense RNA probes were produced. Anti-sense probe was hybridized with mRNA in tissue, whereas the sense probe served as a negative control.

Tissues for in situ hybridization were fixed in 4% paraformaldehyde containing sucrose, frozen in liquid nitrogen and cut into 10 µm-thick sections using a cryostat. The sections were then placed on 0.1% polylysin (sigma)-coated slides. Prior to hybridization, the specimens were hydrated in PBS, incubated with Triton X-100 (0.3% in PBS), treated with proteinase K (1 µg/ml Tris-EDTA buffer) and post-fixed using 4% paraformaldehyde. To reduce non-specific binding, the specimens were treated with 10 mM iodoacetamide and 10 mM N-ethyleneimide. The specimens were pre-hybridized for 2 h at 42°C in 2 X SSC solution containing 50% formamide. Twenty microliters of hybridization mixture were applied to every section. The 20 µl hybridization mixture contained 18 µl hybridization buffer (50% deionized formamide, 5 X Denhardt's solution, 10% dextran sulfate, 0.5% sodium pyrophosphate, 0.5% SDS and 250 µg/ml denatured salmon sperm DNA) and 2 µl probe solution (radioactivity more than $0.8X10^6$ cpm). The hybridization was carried out in a moist chamber for 16 h at 42° C. To remove unhybridized RNA, sections were washed in RNase-

containing (20 µg/ml) 2 X SSC solution for 30 min at 42°C. Posthybridization washing was performed for about 2 h at 40° C in a 0.1 X SSC solution with several changes of fresh solution. After washing, the specimens were dehydrated in 70 %, 90%, and 100% ethanol containing 0.3M ammonium acetate and dried. The sections were then immersed in an emulsion (LM-1 Amersham) and exposed for 10 days at 4°C in the dark. Finally, the autoradiograms were developed, fixed and counter-stained by hematoxylin for further examination.

4.3.5. Statistics

The statistical analysis was performed using ANOVA and Tukey's Honest Significant Difference (HSD) test was applied for multiple comparison, employing the SYSTAT software package (Systat inc. Evanston, IL). The *p* values less than or equal to 0.05 were considered significant.

4.4. Results

NORTHERN BLOT ANALYSIS

Northern blots were prepared from cytoplasmic RNA obtained from normal rabbit aortic tissue or from neointimal aortic tissues 4-, 8- and 12-weeks after the initial arterial deendothelialization. Cytoplasmic RNA from human fibroblasts was used as reference for the size and concentration of mRNA of MMPs and TIMP-1.

Rabbit MMP-9 mRNA was a transcript of 2.8 kb. In normal aortic tissues, the signal for this mRNA was quite weak, reflecting the absence or a very low expression of this gene. In contrast, strong signals as evidenced by high intensity bands, were observed from mRNA isolated from neointimal tissues at the 4th, 8th or 12th week after arterial injury (Fig.1A). The intensities of signals from different tissues were measured using a densitometer and normalized to the signal for mRNA of α-tubulin. As shown in Fig. 1B, the MMP-9 signals of neointimal tissues, developed at 4, 8, and 12 weeks, were not significantly different.

Signals for MMP-2 mRNA, transcripts of 3.2 kb, were present in both normal and neointimal tissues. However, the intensities of signals were high in neointimal tissues taken at the 4th or the 8th week after injury, returning to normal the 12th week after injury (Fig. 2A). Quantitative assesment showed that the mRNA expression in neointimal tissues isolated at the 4th or the 8th week was 2 fold higher ($p<0.05$) than in the normal tissues or neointimal tissues at 12 weeks after injury (Fig. 2B).

Rabbit TIMP-1 mRNA was a transcript of 0.9 kb. Fig. 3A shows that signals for this mRNA were present in both normal and neointimal tissues, although its signal in normal arterial tissue was weak.. Furthermore, the expression of this gene in neointimal tissues appeared to progress with time after deendothelialization. TIMP-1 signals at the 4th, 8th and 12 th week were 10-, 15-, and 18-fold

stronger respectively in neointimal tissues.(Fig. 3B). Thus neointimal tissues synthesized significantly more TIMP-1, although the difference between tissues, isolated at different intervals after surgery, was not statistically significant.

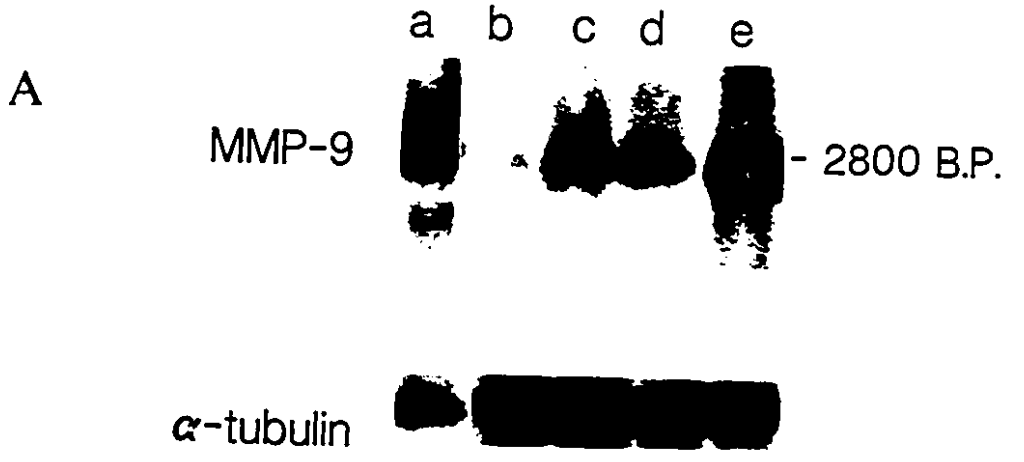
IN SITU HYBRIDIZATION

The in situ hybridization experiment visualized the spatial localization of cells expressing mRNA for MMP-2, MMP-9 and TIMP-1 in *ex vivo* preparations. The histological characteristics of neointimal tissue after a balloon catheter-induced deendothelialization were similiar to those described before, and immunostaining with SMC specific α-actin confirmed that the majority of neointimal cells were SMCs.

No positive signal for mRNA of MMP-9 was seen in normal arterial tunica media using ^{35}S-labeled single-strand antisense cRNA probes. However, signals for MMP-9 were quite strong in the neointimal tissues. These signals were uniformly distributed in the whole layer of neointima and were similar at 4, 8 and 12 weeks (Fig 4). Figure 5 showed the expression of mRNA of MMP-2 in normal tunica media and neointimal tissues. Northern blot analyses indicated a uniform MMP-2 distribution.

It should be emphasized that in situ hybridization, in contrast to Northern blot analysis, could not detect mRNA signal for TIMP-1 in normal tunica media. In the neointimal tissues taken at 4-, 8-, and 12-week after deendothelialization, TIMP-1 signals were strong and uniformly distributed in neointimal tissues (Fig. 6).

No positive signals were seen in sections incubated with sense probes for MMP-2, MMP-9 and TIMP-1.



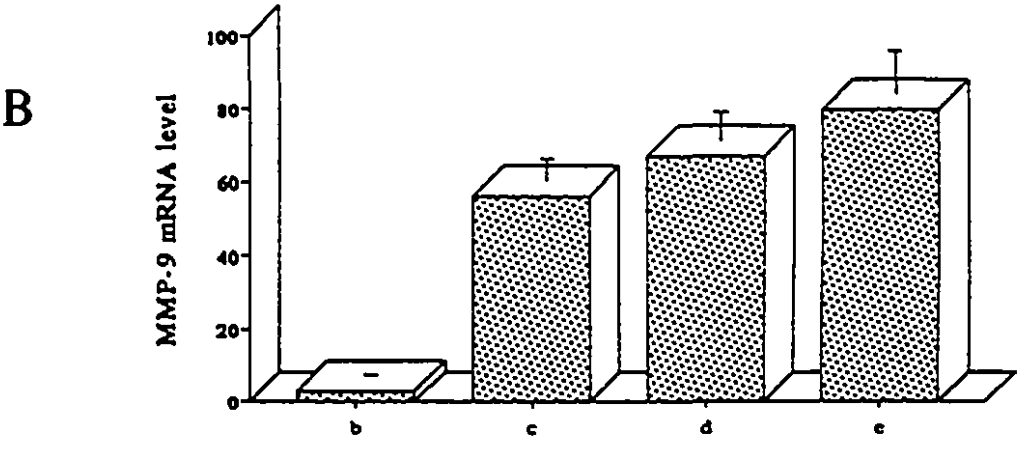


Fig. 1. Analysis of MMP-9 mRNA expression. Panel A: A representative Northern blot of cytoplasmic RNA (20 μg/lane). a: positive control; b-e: samples from normal and neointimal (at the 4th, 8th, and 12th week after arterial de-endothelialization) arterial tissues respectively. Panel B: Graphic illustration of the densitometric measurement of MMP-9 signals. Values are mean ± SD (4th week, 58 ± 3.5; 8th week, 66 ± 5.0; 12th week, 72 ± 8.6. n=4).

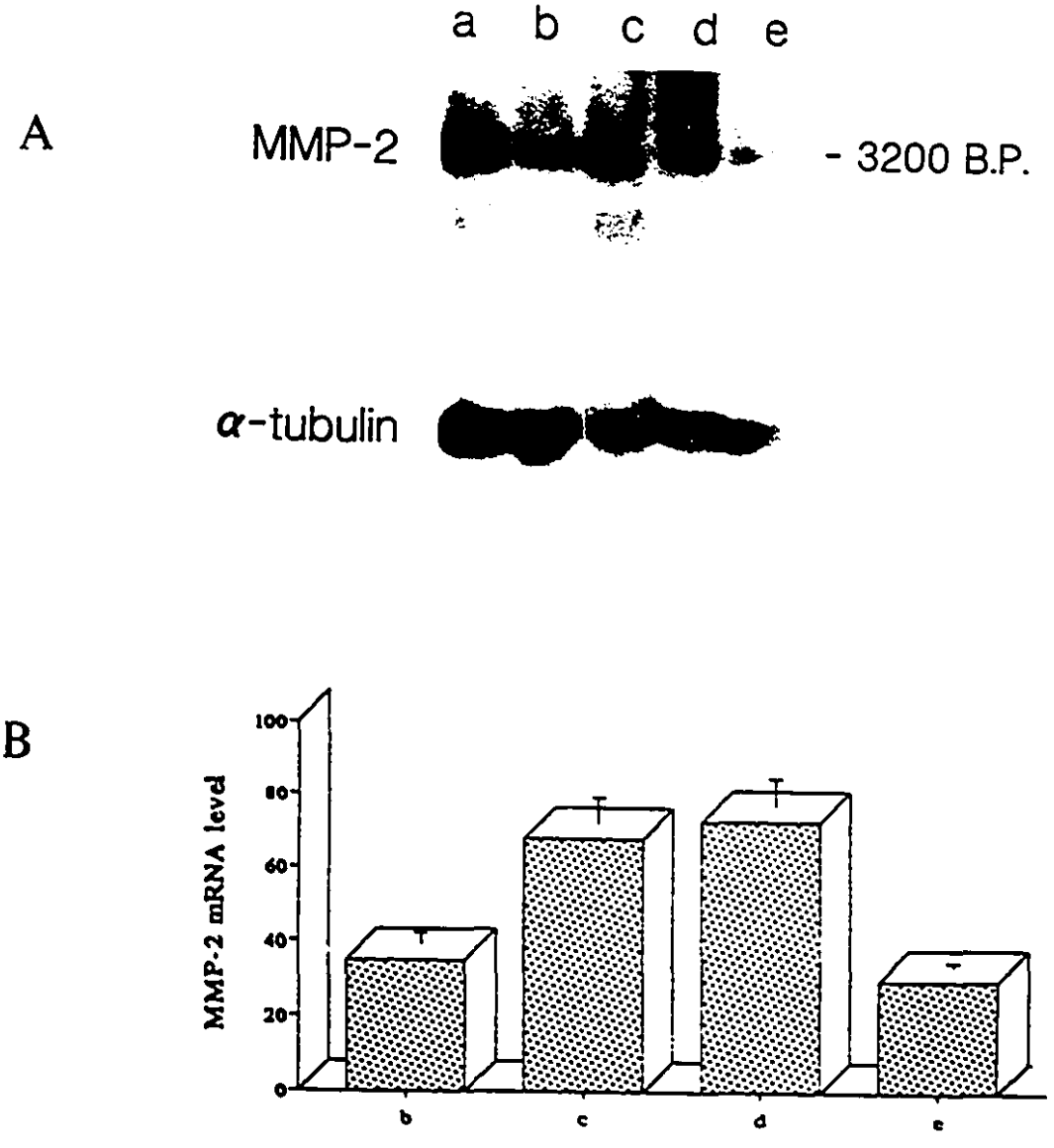
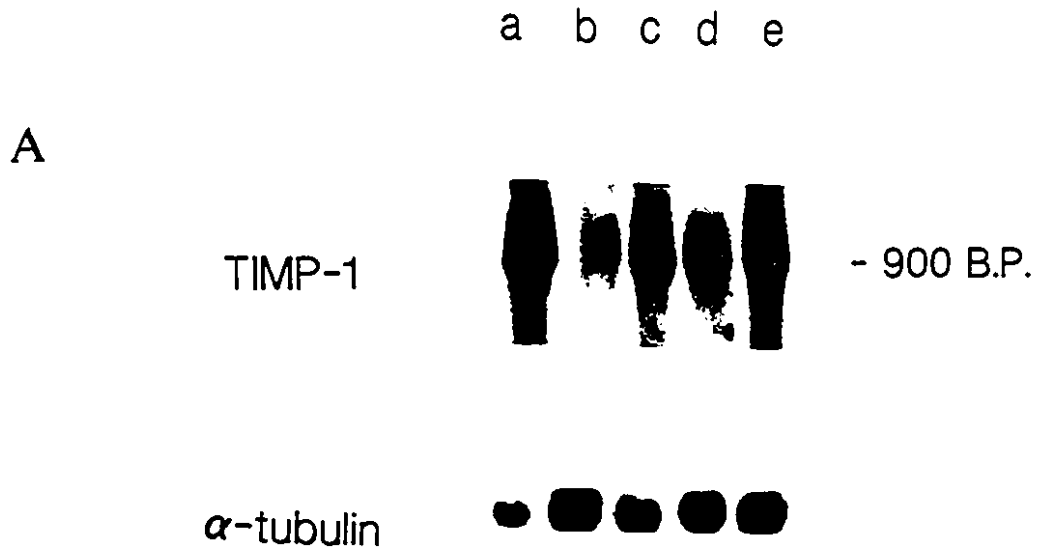


Fig. 2. Analysis of MMP-2 mRNA expression. Panel A: A representative Autoradiograph of a Northern blot. a: positive control. b-e:samples from normal and neointimal (at the 4th, 8th, and 12th week after arterial de-endothelialization) arterial tissues respectively Panel B: Graphic illustration of the densitometric measurement. Values are mean ± SD (normal arterial tissue, 38 ± 3.1; 4th week, 67 ± 5.1; 8th week, 74 ± 6.0; 12 weeks, 29 ± 2.5. n=4).



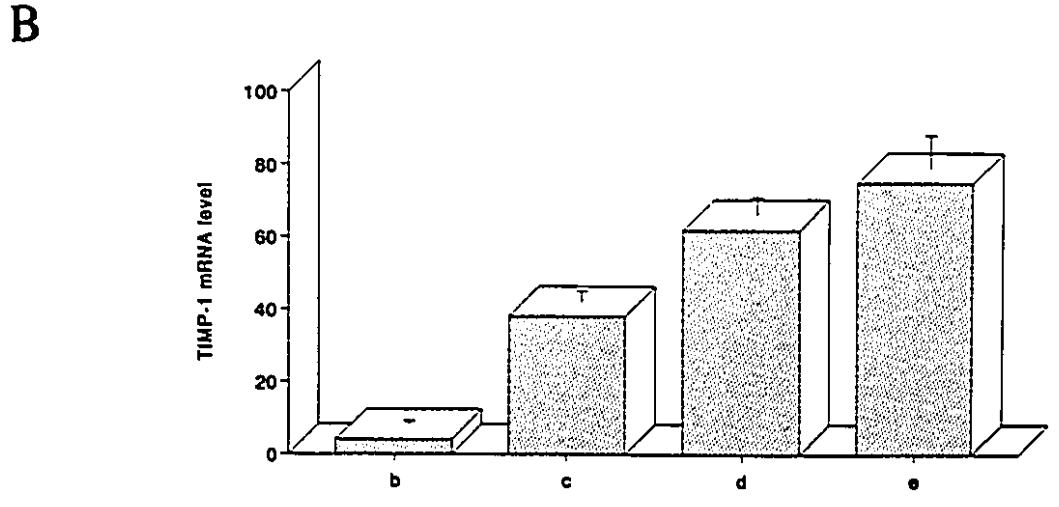
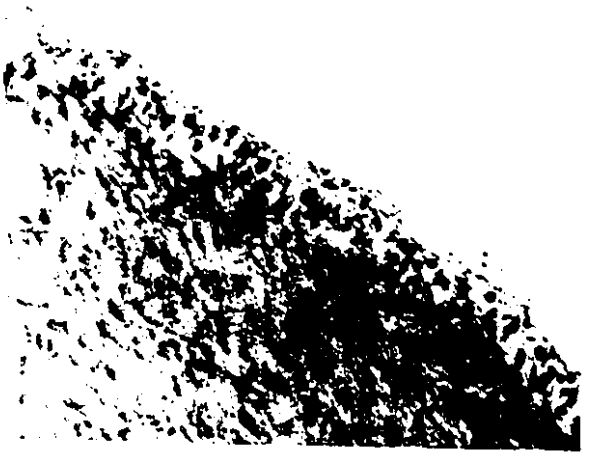


Fig. 3. Analysis of TIMP-1 mRNA expression. Panel A: A typical Northern blot using ^{32}P-labeled huamn TIMP-1 cDNA. a: positive control. b-e:samples from normal and neointimal (at the 4th, 8th, and 12th week after arterial de-endothelialization) arterial tissues respectively Panel B: Graphic illustration of the densitometric measurement Values are mean ± SD (normal arterial tissue, 4.0 ± 0.9; 4th week, 38 ± 1.9; 8th week, 62 ± 4.6; 12th week, 75 ± 5.7. n=4).

a



b

c

Fig. 4. In situ hybridization of deendothelialized rabbit aortic tissue with ^{35}S-labeled RNA probes for MMP-9, counterstained with hematoxylin. A and B: neointimal tissues developed 4 and 12 weeks after arterial injury. C: negative control with sense probe. Magnification X200 for A and C; X250 for B.

a

b

c

Fig. 5. In situ hybridization of deendothelialized rabbit aortic tissue with ^{35}S-labeled RNA probes for MMP-2, counterstained with hematoxylin.A: neointimal tissue 8 weeks after injury; B: neointimal tissue 12 weeks after injury; C: negative control with sense probe. Magnification X200 for A and C; X300 for B.

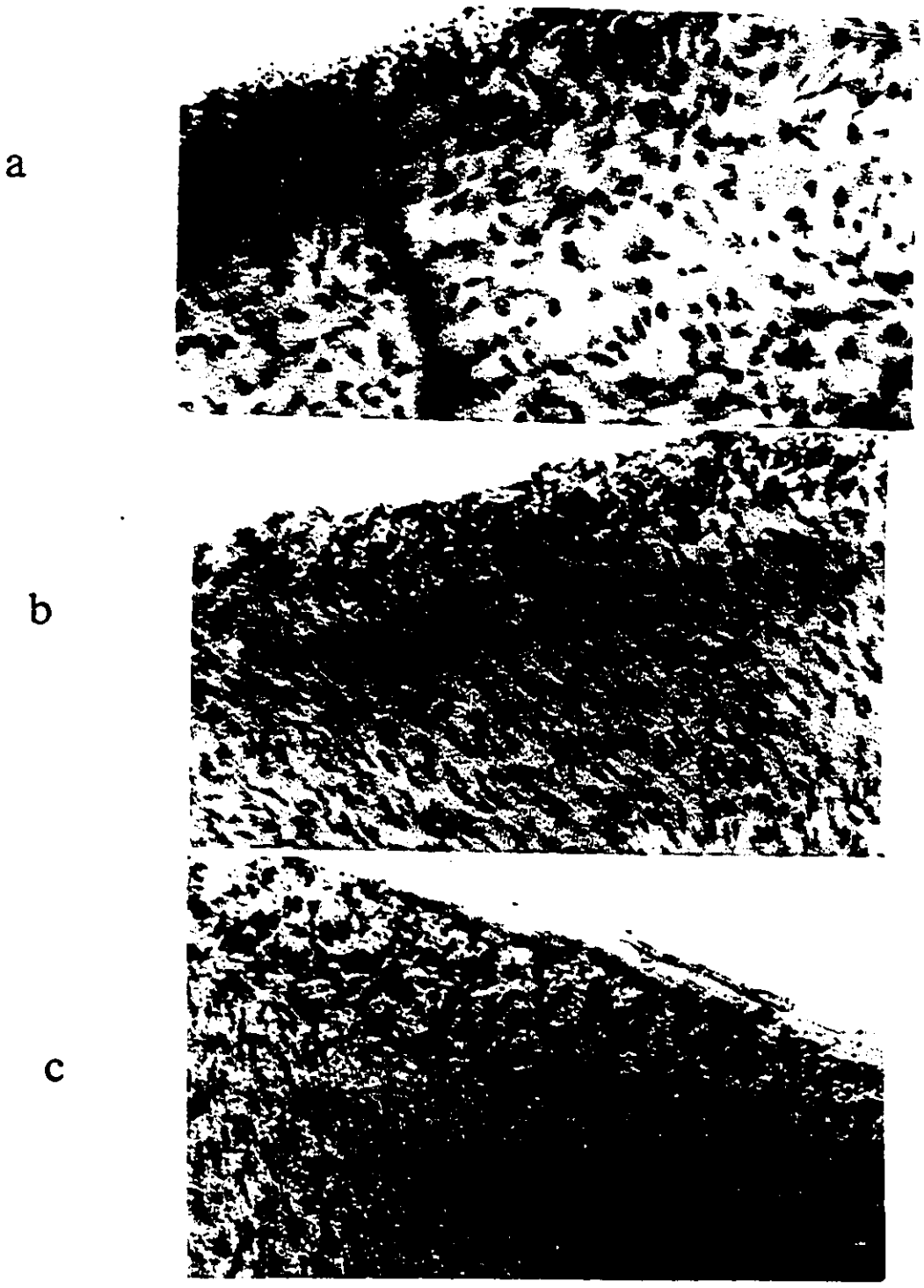


Fig. 6. In situ hybridization of deendothelialized rabbit aortic tissue with ^{35}S-labeled RNA probe for **TIMP-1**, counterstained with hematoxylin. A,B and C: neointimal tissues developed 4, 8 and 12 weeks after arterial injury. Magnification X200.

4.5. Discussion

We report that expression of mRNA for MMP-2 and MMP-9 appears to increase in neointimal SMCs: this injury induced increase in mRNA expression seemed to continue at least for 8 weeks for MMP-2 and for 12 weeks for MMP-9. In neointima, MMP positive cells were distributed uniformly and immunohistochemical staining indicated that the majority of these cells were SMCs. This is in agreement with reports which suggested that MMP-2 and MMP-9 are involved in SMC proliferation and migration.[5,15] Furthermore, the present data indicate that the synthesis of MMPs in neointimal lesions is a relatively prolonged process. Also arterial SMCs are a major source of arterial MMPs.

Migration and proliferation of SMC, has long been implicated in atherogenesis and is considered to facilitate lipoprotein retention.[20, 21] In the arterial wall, SMCs are usually surrounded by ECM components, such as collagen, heparan sulfate proteoglycan (HSPG), elastin and fibronectin, which provide resistance to their migration. A large portion of these ECM components can be degraded by MMP-2 and MMP-9. There is evidence that balloon catheter deendothelialization could cause phenotypic changes of arterial SMCs and increase their production of certain growth factors and ECM components. These phenotypic changes could influence MMP metabolism and may be responsible for enhanced mRNA expression in this study. The MMPs might have facilitated degradation of ECM, releasing the SMC to migrate from media to intima, compatible with the observation of Pauly et al.[22], who showed that selective neutralizing antibody to MMP-2 can inhibit the migration of SMC across a synthetic ECM membrane. Besides facilitating migration, increased MMP activity may also promote the proliferation of SMCs. Proliferation of SMC can be mediated by two pathways. First, MMPs may degrade ECM components like HSPG, which usually inhibit

proliferation. Second, certain growth factors in the arterial wall, such as bFGF, could be detached from matrix by the action of MMPs and become readily available to induce cellular proliferation.[23]

The expression pattern of MMP mRNA in arterial SMCs is determined by 1) promoter sequences of the genes 2) environmental cytokines 3) surrounding ECM components 4) organization of the cytoskeletal system. The MMP-2 gene promoter has the characteritic of a housekeeping, or constitutive promoter, explaining its expression in normal arterial SMCs. The promoter of MMP-9, on the other hand, has an AP-1 binding site, accounting for its strong and lasting reaction to induction. Cytokines are another important regulator of MMP transcription. Previously, we and others[24] have shown the participation of a variety of cytokines, including IL-1 and TGF-β, in the development of balloon catheter-induced arterial neointima. IL-1 can induce the production of protein product encoded by the *fos* and *jun* oncogene families. These proteins in turn attach to the AP-1 binding site in MMP-9 promoter and start MMP-9 gene transcription. Cytokines can also influence MMP gene expression through the transduction of cellular surface receptors.[25] Both TGF-ß and IL-1 may activate MMP-2 gene transcription through the Ca^{++}-protein kinase C pathway.[26] Besides these regulatory pathways, reports have indicated that laminin, an ECM component, can increase the expression of MMP-2 mRNA,[27,28,29] and the loss of actin polymerization results in decreased expression of mRNA of MMP-9.[30,31,32] The intracellular signaling pathways for these ECM components are not well understood.[9]

Increase of TIME-1 synthesis by neointimal SMCs, another finding of this study, is similar to the situations described in human osteoarthritis and rheumatoid arthritis.[12] While the role of increased TIME-1 in atherogenesis needs further investigation, in situ hybridization results showed that TIME-1

was adjacent to MMP, suggesting a role in inhibiting MMP activity. The time course analysis of this inhibitor showed that the magnitude of TIMP-1 increase is relatively low in the early development of the neointima and becomes more pronounced in the later phase of development. The low level in the early stage generally favors MMP activity and hence, migration and proliferation of arterial SMCs. Pronounced TIMP expression in the later stage tends to balance MMP activity, in favor of ECM accumulation in the arterial lesions. Nonetheless, even in this stage, one cannot exclude the possibility of some MMP activity in certain areas, such as those rich in macrophages.[33] Besides inhibiting MMP activity, increased TIMP-1 concentration in neointimal tissue may also contribute to proliferation of cells, as TIMP-1 has been shown to promote proliferation of fibroblasts and endothelial cells.[34]

Elevation of TIMP-1 in our model may, again, be attributed to the actions of numerous mitogens in the arterial neointima, including PDGF, EGF, bFGF, TGF-ß and IL-6.[17] These growth regulatory factors can induce TIMP-1 overproduction through activation of protein kinase C signal transducing pathways or through their interaction with special sequences in the TIMP-1 gene, such as AP-1 and PEA3.[35]

4.6. Acknowledgments

We are grateful to Dr. M. Elizabeth Fini (Department of Ophthalmology, New England Medical Center, Boston. MA) for the generous gift of plasmids containing gelatinase A and B cDNAs. Thanks are also due to Dr. Qutayba Hamid and Dr. Serge Jothy of McGill University for the use of the molecular biology laboratory facility. The excellent technical assistance of Mrs. Archana Srivastava is gratefully acknowledged. This work was supported by Medical Research Council of Canada grant # MT 10590.

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Hypothesis IV:

Interactions between MMPs and TIMPs control the extent of ECM deposition in neointima

Chapter 5

Synthesis of Tissue Inhibitor of Metalloproteinase-1(TIMP-1) in Rabbit Aortic Neointima after Selective Deendothelialization

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5.1. Abstract

Altered TIMP-1 synthesis in the arterial wall may be important for the balance between metalloproteinases and their inhibitors, and thus contribute to dysregulated extracellular matrix metabolism in atherosclerotic lesions. To examine this, we cloned the rabbit TIMP-1 gene from aortic neointima, developed in response to a balloon-catheter induced de-endothelialization. The apparent homology of cDNA with TIMP-1 genes from several sources suggested that it is a rabbit form of TIMP-1. We examined the recombinant rabbit TIMP-1 expression in *E. coli* using pTrxFus expression system and the synthesis of resulting soluble protein was confirmed by immunostaining with anti-TIMP-1. The TIMP-1 concentration in normal and de-endothelialized rabbit aortas was compared using Northern blot, Western blot and mRNA in situ hybridization techniques. We observed a significant increase of TIMP-1 expression in neointimal SMCs at both nucleic acid and protein levels, suggesting a role of TIMP-1 in injury-induced atherogenesis.

Key Words: TIMP; Extracellular matrix; Injury; Atherosclerosis; Gene expression; in situ hybridization; RT-PCR

5.2. Introduction

Extracellular matrix (ECM) metabolism appears to play a pivotal role in the pathogenesis of atherosclerosis [1]. Increased biosynthesis of ECM by proliferating smooth muscle cells (SMCs) has long been known to be responsible for the development of a thickened intima, a well-known early feature of atherogenesis [2]. There is empirical evidence that the increased ECM content facilitates interaction with circulating lipoprotein, resulting in lipid accumulation, a hallmark of atherosclerotic lesions [3,4]. Also, the degradation of ECM is found to be associated with plaque rupture, commonly occurring in advanced atherosclerosis [5].

The degradation of ECM depends largely on proteinases including a family of Zn^{++}-containing enzymes described as "matrix metalloproteinases (MMP)" [6]. The MMPs are present in an inactive (latent) form, pro-MMPs, that require activation before they can exert their biological function [7]. While individual members of the MMP family require specific substrates, collectively the MMPs can digest all of ECM at neutral *p*H [8]. It is also known that activity of MMPs is highly regulated in nature. The degradation of ECM by MMPs can be arrested by specific inhibitors [9]. In recent years, the structure and function of MMP inhibitors have been extensively studied and a family of tissue inhibitors of metalloproteinases(TIMP) has been recognized [10]. The participation of the inhibitors in ECM degradation has also been reported [11].

To date, three types of TIMP, classified as TIMP-1, -2, and -3 , have been characterised in humans and mammals and their expression in a variety of tissues and cells has been reported [12,13,14]. All three TIMP types possess a unique tertiary structure having two distinct 3-loop domains, one on the carboxyl terminal and the other on the amino terminal. This tertiary structure is considered essential for biological function of TIMP [15,16]. A widely studied TIMP is human TIMP-1, which is a glycoprotein of 28.5 KDa and its gene has already been cloned and sequenced [7]. In addition, Huebener *et al.* found that this gene is localized on chromosome X [17]. TIMP-1 from several other species has also been studied [10,13,14,18]. These studies indicated that TIMP-1 is highly conserved among species.

Of particular interest is TIMP-1 synthesis in arterial SMC, because of SMC's role in the pathogenesis of atherosclerosis. Synthesis of TIMP-1 protein by cultured SMC has been reported [19] and its presence in human arterial SMC has been revealed employing immunohistochemistry [20]. Little is known about TIMP-1 modulation at the molecular level.

To investigate this further, we cloned the TIMP-1 gene, examined its sequence and measured its synthesis by Northern analysis of mRNA which was isolated from SMC of the neointima, developed in response to a balloon catheter-induced deendothelialization of rabbit aorta. Our results indicated an enhanced TIMP-1 synthesis in developing neointima.

5.3. Materials and methods

5.3.1. Animals and surgical procedure

New Zealand, white, male rabbits, 2.0-2.5 kg body weight, were acclimatized to the animal quarters for at least two weeks. The surgical procedure to remove endothelium, identical to that reported previously [21] , was performed on a group of ten rabbits. Briefly, the animals were anaesthetized with pentobarbital (30 mg/kg b.wt.). One femoral artery was exposed and a small incision was made. Then a 4F Fogarty, arterial, embolectomy catheter (American Edwards Laboratories, Santa Ana, Calif., USA) was introduced through the incision up to the arch of aorta. The balloon catheter was inflated with 0.75 ml saline and withdrawn with decreasing pressure. The procedure was repeated three times to ensure complete deendothelialization. The catheter was then removed and the incision was closed. Another group of ten rabbits, used as controls, were subjected to a sham operation, i.e. the femoral artery was exposed but no catheter was inserted. Animals of both groups were maintained on normal rabbit chow and water *ad libitum* for the ensuing 8 weeks.

All animal procedures carried institutional approval according to guidelines set by the Canadian Council on Animal Care.

5.3.2. Tissue preparation

The rabbits were killed 8 weeks after balloon-catheter de-endothelialization. Thirty minutes before killing, the animals were injected with 5 ml of a 0.45 % solution (wt/vol) of Evans blue dye (Allied Chemical Company, New York, New York), which outlines with precision the areas where endothelium has not regenerated (deendothelialized area, DEA) by staining them blue, whereas the

areas where endothelium has regenerated (reendothelialized area, REA) appeared white on a blue background. At sacrifice, the aortas from arch to bifurcation were removed *en bloc* and then were opened longitudinally. For in situ hybridization, tissues from normal or reendothelialized areas were selected and fixed immediately. For RNA extraction and protein analysis, the endothelium was removed from the aorta and the intima-media layer was peeled from the adventitia using Bergh cilia forceps as described elsewhere [21].

5.3.3. RNA extraction and cDNA synthesis

Cytoplasmic RNA was extracted using TRIzol Reagent (GIBCO BRL) according to Chomcynski's method [22]. RNA thus obtained was further purified by digesting with DNase (Promega) for 60 min at 37°C. The purified RNA was used for cDNA synthesis and Northern blot analysis.

For cDNA synthesis, an aliquot of 15 µg purified RNA was added into 50 µl aqueous solution containing 300 units SuperScript II Reverse Transcriptase (BRL), 60 units RNasin (Promega), 25 µg random hexanucleotide primers (Amersham), 5 µl 10 X reverse transcription buffer (BRL) and 1.25 mM dNTP. The synthetic reaction was carried out for 60 min at 42°C.

5.3.4. RNA analysis

TIMP-1 mRNA expression was studied using Northern blot analysis. Cytoplasmic RNA was separated on 1% agarose gel containing 0.6 M formaldehyde and then transferred onto a nylon membrane (Hybond-N+, Amersham) using a semi-dry 2117 MULTIPHOR II electrophoresis unit (LKB). The membrane, with immobilized RNA (by UV cross-linking), was hybridized to a TIMP-1 cDNA probe. This probe was prepared from a plasmid containing human TIMP-1 cDNA (A gift from Dr. Donald Brown , Ophthalmology Research Laboratory, Cedars-Sinai Medical Center, CA). Radiolabeling of this probe was done using [α-^{32}P]-dCTP (ICN) and T7 random labeling kit (Pharmacia). Autoradiography was performed by exposing X-ray film (Kodak X-Omat AR) and intensifying screens for 2 weeks at -70°C. Autoradiograms were scanned using a Hoefer GS 3000 densitometer (LKB) to measure the TIMP-1 signal with α-tubulin hybridization as a reference for relative mRNA load per lane.

5.3.5. Characterization of cDNA for TIMP-1

The cDNA encoding TIMP-1 core protein was amplified using polymerase chain reaction

(PCR). A typical PCR cycle consisted of: denaturation (1 min at 96°C), annealing (1 min at 57°C) and extension (2-3 min at 72°C). Forty PCR cycles were carried out in the presence of 1.5 units Taq DNA polymerase (Promega) to get sufficient cDNA copies. Oligonucleotide primers were synthesized by the Sheldon Biotechnology Centre of McGill University and restriction endonuclease sites were placed at both ends of the primers (Table 1). The PCR products were directly purified from the gels and subcloned into pGEM-T plasmid vector (Promega).

Table 1: *Oligonucleotide primers used for TIMP-1 amplification*

name	Sequence	Orientation
S1	5'-GGATTCACCATGGCCCCCTTGGC	Sense
S2	5'-CCGGATCCTGCACCTGTGTCCCACCTCACCCGCA	Sense
A1	5'-GAGCAGCCTTCAGTCTTTCCGGGGCCGCAGGGACT	Antisense

The subcloned cDNA fragments were sequenced by the dideoxy chain termination method using an automatic DNA Sequencer (Pharmacia) and fluorescent primers (T7 and SP6). BLAST Server of NIH was used for sequence comparison.

5.3.6. Expression and purification of rabbit TIMP-1 protein

An aliquot of subcloned TIMP-1 cDNA was inserted into bacterial expression vector pTrxFus (Invitrogen). In this vector, TIMP-1 was expressed as a fusion protein with bacterial protein thioredoxin (trxA). This expression was under the control of a Pl promoter. The pTrxFus was propagated in GI 698 (an *E. coli* strain). A *trp* promoter in GI 698 put TIMP-1 translation under the control of tryptophan.

An aliquot of GI 698, with the recombinant pTrxFus, was grown at 15°C in a culture medium called RM (Invitrogen) which contains M9 salts, Casamino acids, glycerol, MgCl2 and ampicillin. When the bacteria reached the midlog phase, tryptophan (final concentration, 100 μg/ml RM) was added to induce TIMP-1 expression and the incubation temperature was raised from 15°C to 30°C.

Extraction of the expressed protein was performed as described by LaVallie *et al.* [23] with slight modification. Briefly, cultured bacteria were precipitated and resuspended in 10 mM Tris

buffer (in a volume of 1/100 original culture medium). The suspension was sonicated for 30 sec, heated for 2 min at 80°C, snap frozen, thawed and centrifuged at 4°C. After centrifugation, most TIMP-1 remained in the supernatant. Purification of TIMP-1 was done by passing the supernatant through 2 chromatographic columns in series. The first column was a sepharose CL-6B (Pharmacia, LKB) column, pre-equilibrated and eluted with 10 mM Tris buffer. The second column was a ThioBond resin column (Invitrogen), pre-equilibrated and eluted with 10 mM Tris buffer containing 20 mM ß-ME. After chromatography, eluents were pooled, concentrated and dialyzed against 10 mM Tris buffer containing proteinase inhibitors (EDTA, benzamidine-HCl and α-aminocaproic acid). The protein preparation was subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel in order to resolve TIMP-1 into a specific band. The TIMP-1 was identified by immunoblotting with anti-human TIMP-1 (Cambio, Millington Road, Cambridge CB3 9HP).

5.3.7. In situ hybridization analysis

a. Preparation of probe

A plasmid vector containing rabbit TIMP-1 was prepared as described above. The plasmid was linearized with restriction enzymes. RNA probes were generated either with T7 or with SP6 polymerase (Amersham) and radiolabeled with [α-^{35}S]-UTP. The specific activity of these probes were usually 1.5X10^{6} cpm/ng RNA. Both sense and anti-sense RNA probes were produced. The anti-sense probe hybridized with mRNA in tissue and the sense probe served as a negative control.

b. Hybridization procedure

Tissues for in situ hybridization were fixed in 4% paraformaldehyde containing sucrose, frozen in liquid nitrogen and cut into 5 µm-thick sections using a cryostat. The sections were then placed on 0.1% polylysin (Sigma)-coated slides. Prior to hybridization, the specimens were hydrated in PBS, incubated with Triton X-100 (0.3% in PBS), treated with proteinase K (1 µg/ml Tris-EDTA buffer) and post-fixed using 4% paraformaldehyde. To reduce non-specific binding, the specimens were treated with 10 mM iodoacetamide and 10 mM N-ethyleneimide. The specimens were pre-hybridized for 2h at 42°C in 2 X SSC solution containing 50% formamide. Twenty microliters of hybridization mixture was applied to every section. The 20 µl hybridization mixture contained 18 µl hybridization buffer (50% deionized

formamide, 5 X Denhardt's solution, 10% dextran sulfate, 0.5% sodium pyrophosphate, 0.5% SDS and 250 μg/ml denatured salmon sperm DNA) and 2 μl probe solution(radioactivity more than $0.8X10^6$ cpm). The hybridization was carried out in a moist chamber for 16h at 42°C. To remove unhybridized RNA, sections were washed in RNase-containing (20 μg/ml) 2 X SSC solution for 30 min at 42°C. Posthybridization washing was performed for about 2h at 40°C in a 0.1 X SSC solution with several changes of fresh solution in between. After washing, the specimens were dehydrated in 70 %, 90%, 100% ethanol containing 0.3M ammonium acetate and dried. The sections were then immersed in an emulsion (LM-1 Amersham) and exposed for 10 days at 4°C in the dark. Finally, the autoradiograms were developed, fixed and counter-stained by hematoxylin for further examination.

5.3.8. Protein analysis

The quantity of TIMP-1 protein was measured by immunoblotting. Briefly, the aortic tissues were homogenized in the presence of TRIzol (1ml/100mg tissue) and chloroform (0.2 ml/ ml TRIzol). The homogenate was then incubated for 5 min and centrifuged. The centrifugation resulted in phase separations: the upper aqueous phase contained RNA; intermediate phase contained DNA; the lower organic phase contained protein. The aqueous phase was removed for RNA extraction. The intermediate DNA was further purified as described by Chomczynski [24]. The organic phase, with its protein, was dialized for 72h at 4°C against 0.1% SDS aqueous solution containing proteinases (EDTA, benzamidine-HCl and α-aminocaproic acid). After dialysis, protein preparation, corresponding to 5 μg of DNA, was mixed with an equal volume of sample buffer which contained 5% SDS, 2% mercaptoethanol and 2% bromophenol blue. After 10 min boiling in a water bath, the reduced samples were electrophoresed on a 7.5% SDS-polyacrylamide gel. Protein was transferred to a nitrocellulose membrane (Bio-RAD Laboratories, Richmond, CA 94804) using a semi-dry 2117 MULTIPHOR II electrophoresis unit (LKB) as described in an earlier paper [25]. Membrane was incubated with 1:2000 diluted polyclonal anti-TIMP (Cambio, Millington Road, Cambridge CB3 9HP). Mouse anti-rabbit IgG conjugated to biotin was used as the secondary antibody. Detection of TIMP-1 protein was accomplished by densitometry using a Hoefer GS 300 scanning densitometer equipped with GS-360 densitometry software [3].

5.4. Results

Northern Blot analysis

Rabbit TIMP-1 mRNA was a transcript of 0.9 kb. As shown in figure 1, the signal for this mRNA was present in both normal and neointimal SMCs. However, the signal was much more intense in the neointimal SMC. Indeed, TIMP-1/α-tubulin mRNA signal in neointimal SMC was 15-fold stronger ($p < 0.01$) than in control SMC (Fig. 2).

Molecular cloning and expression of rabbit TIMP-1 cDNA

The cDNA clones, encoding rabbit TIMP-1 core protein, were obtained following amplification of primers A1 and S1 using PCR technique. A prominent cDNA band of 650 base pairs (Fig. 3) was found. The hybridization of this cDNA with human TIMP-1 produced an intense signal indicating that it probably is TIMP-1.

Further analysis was done by sequencing of this cDNA after subcloning into a plasmid vector. As shown in figure 4, this TIMP-1 contained 12 cysteine residues, 2 potential sites for-N-linked glycosylation and a hydrophobic signal peptide. This was similar to the sequence reported for rabbit pulmonary TIMP with the exception of a single base substitution (C-T) at 375 bp, without affecting the amino acid sequence. There appeared to be close homology in TIMP isolated from different species.

Figure 5 showed the TIMP-1 expression in a bacterial system. Insertion of cDNA resulted in the production of a 32 KDa soluble protein, which had strong immunostaining with anti-TIMP-1.

In situ hybridization

In order to investigate the spatial localization and to identify the cellular source(s) of TIMP-1 mRNA in vivo, we utilized the mRNA in situ hybridization technique. In normal arteries, only endothelial cells gived a positive signal for TIMP-1, and medial cells were usually negative. However, the neointima, developed in response to injury, exhibited a strong TIMP-1 signal (Fig. 6a), that could not be solely attributed to endothelial cells. The majority of the neointimal cells were SMCs as indicated by immunostaining with SMC specific α-actin. The sections incubated with the

sense probe did not have any signal for TIMP-1 (Fig. 6b).

TIMP-1 protein analysis

Figure 7 showed a 4-fold increase in TIMP-1 protein of neointima over control tissues ($p < 0.01$). However, this increment of TIMP-1 protein was far less than the 15-fold increase in neointimal mRNA.

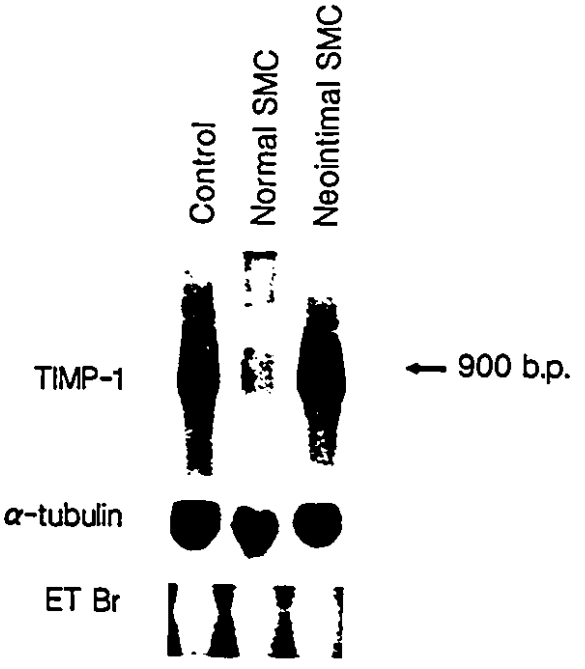

Fig. 1. Northern blot analysis of TIMP-1 mRNA expression in normal and neointimal SMCs. Cytoplasmic RNA from human fibroblasts was used as a positive control. The blot was densitometrically scanned and normalized to α-tubulin for quantification.

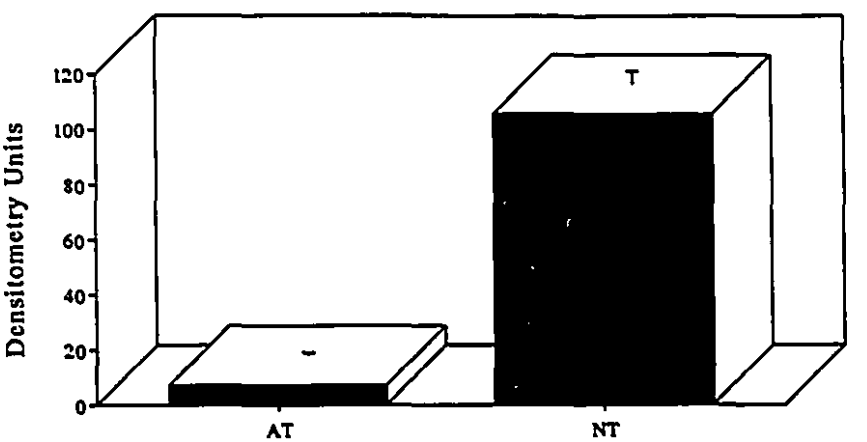

Fig. 2. Bar graph showing signals for TIMP-1 mRNA is significantly increased in neointimal SMC ($p < 0.01$). Values are represented as mean ± SD (normal SMC, 7.1±1.0; neointimal SMC, 106±4.8; n=4). AT, normal aortic tissue; NT, neointimal tissue.

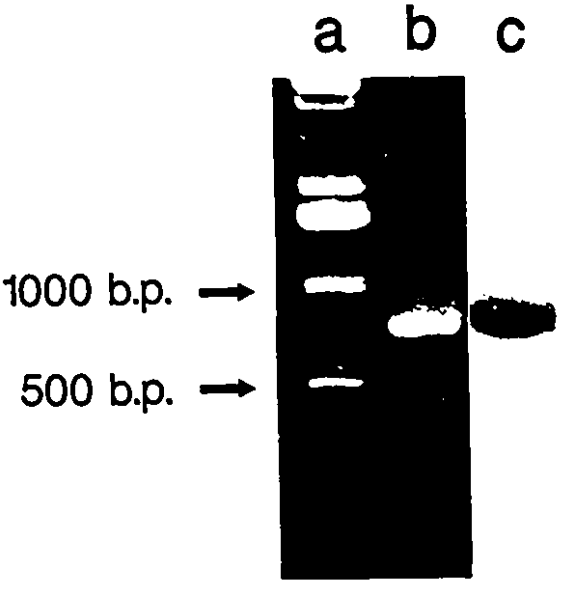

Fig. 3. PCR amplification and hybridization of TIMP-1 sequence from rabbit SMC. Lane b shows an ethidium bromide-stained agarose gel of the PCR products. Lane c shows an autoradiogram of the blot obtained when this DNA was hybridized to a ^{32}P labeled human TIMP-1 probe. Lane a is a molecular weight marker. (b.p.= base pair).

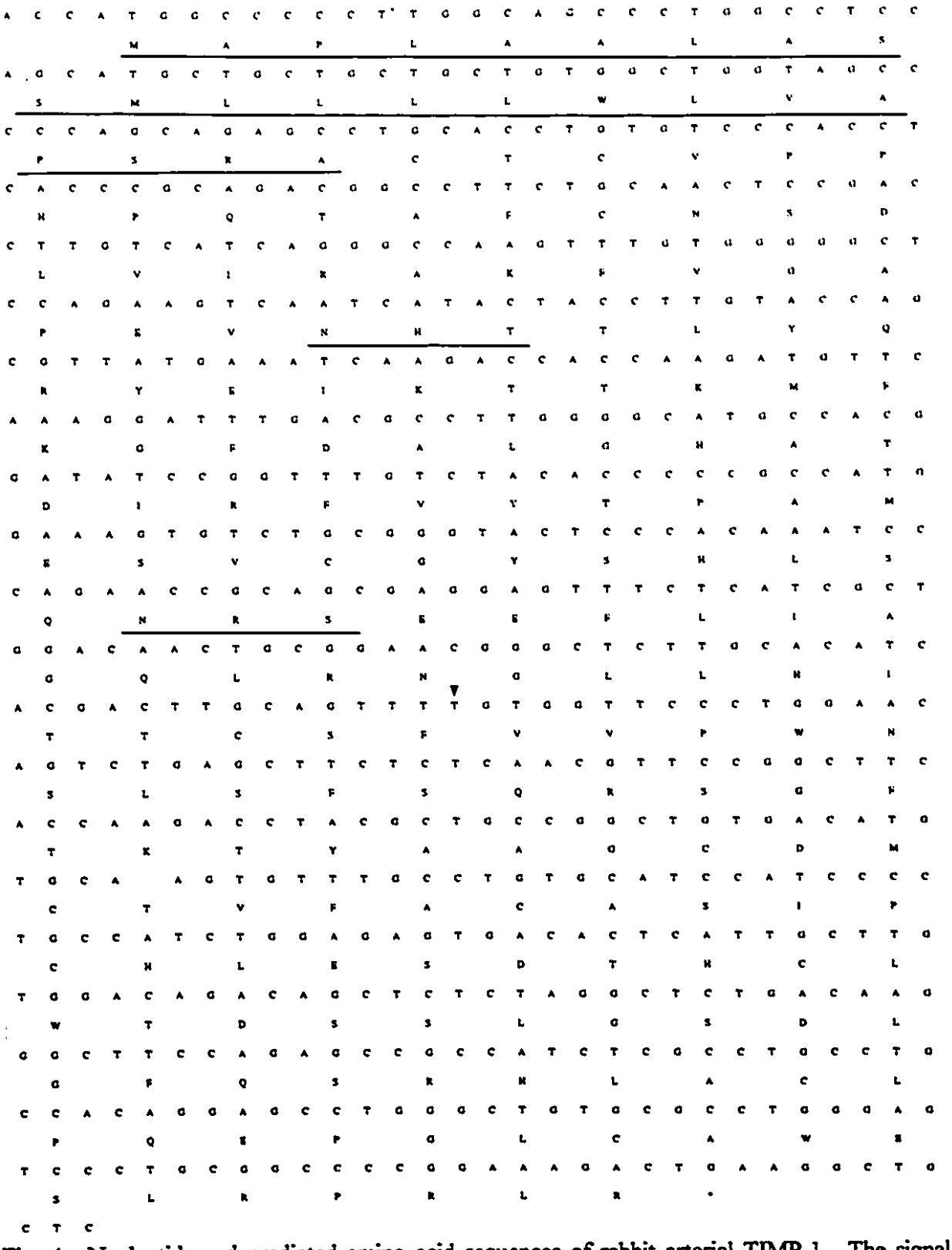

Fig. 4. Nucleotide and predicted amino acid sequences of rabbit arterial TIMP-1. The signal sequence and two potential sites for N-linked glycosylation are underlined. Arrow indicates single base substitutation as compared to reported sequence of rabbit pulmonary TIMP-1. The stop codon is marked with an arrow head.

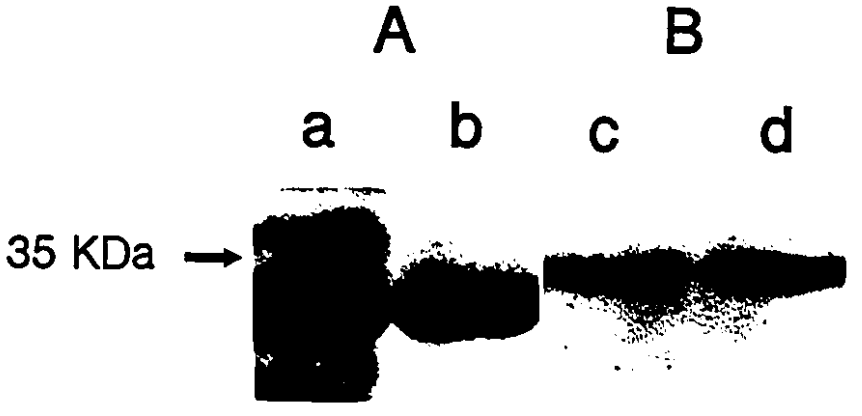

Fig. 5. Expression of recombinant TIMP-1 gene in *E. coli*. Coomassie blue-stained SDS-PAGE gel (A) and corresponding Western blot (B) probed with anti-TIMP-1. Lane a&d: soluble proteins from infected bacteria. Lane b&c: purified TIMP-thioredoxin fusion protein by FPLC and ThioBond resin.

A

B

Fig. 6. In situ hybridization of rabbit aorta by ^{35}S-labeled RNA probes for TIMP-1. The sections were counterstained with hematoxylin. Anti-sense TIMP-1 probe bound mRNA in neointimal SMCs (A, X20), whereas no binding of sense probe was seen (B, X20).

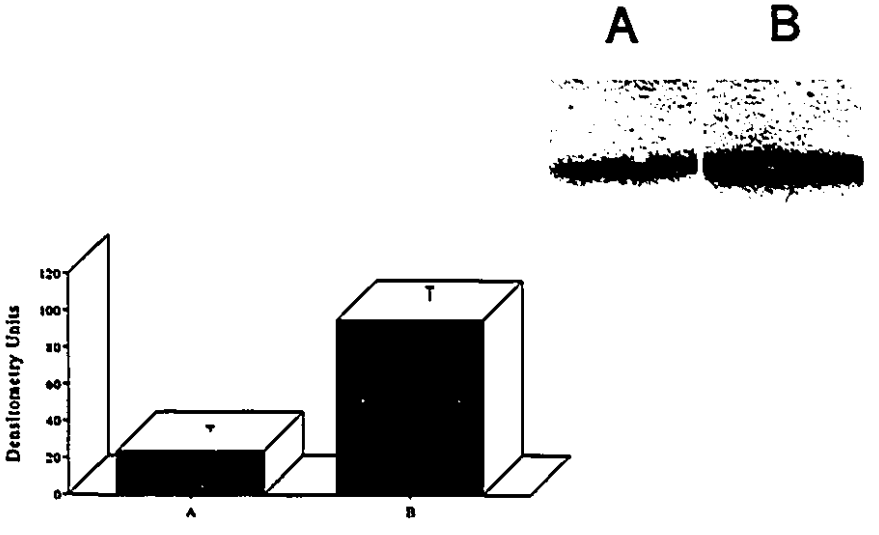

Fig. 7. Bar graph showing TIMP-1 protein in rabbit aorta. The protein level is significantly increased ($p<0.01$) in neointimal tissue as compared to normal tissue (95.2±7.4 vs 23.7±3.0; n=3). A, normal aortic tissue; B, neointimal tissue.

5.5. Discussion:

In the present report, by sequencing and cloning a TIMP-1 gene from rabbit arterial tissue explants, we extended the investigation of TIMP-1 to an organ system. Previous observations were made with cultured bovine cells [19]. The active arterial TIMP-1 was identified by the specific tertiary structure of its protein containing 6 disulfide bonds between 12 cysteine residues. This tertiary structure is essential for TIMP activity, since alteration in this structure, particularly breakage of any disulfide bond, renders it inactive [26]. Also, the presence of specific codons between cysteine 3 and cysteine 13, especially His7 and Gln9, facilitate TIMP-1 identification. The His7 and Gln9 residues of these codons influence TIMP-1 activity. Mutation in these residues destabilizes binding to MMP, thus inactivating TIMP-1 [27]. Further analysis shows that this gene has two sites for N-linked glycosylation, a characteristic feature of TIMP-1 [28]. It is known that the clearance of TIMP depends on sugar chains linked to these sites [29]. A molecular weight of 28.5 KDa is generally reported for TIMP [9] but our recombinant product was found to be 20 KDa. This difference in molecular weight could be due to the bacterial system used for recombinant experiments. Taken together, these structural similarities suggested that the cloned gene was TIMP-1. However, the compelling evidence for the identification was an identical amino acid sequence derived from this gene and that from rabbit pulmonary TIMP-1 cDNA. The comparison between the two cDNA sequences revealed only a single base substitution, namely C for T at position 375 bp, without affecting the amino acid sequence [30].

A second important finding of this study was the clear demonstration of greatly elevated TIMP-1 synthesis both at mRNA and protein levels by the smooth muscle cells, derived from the neointima, developed in response to a selective arterial denudation of rabbit aorta. The reason or

reasons for this elevated synthesis are yet to be understood. However, it can be ascribed, to some extent, to the abundance of several growth factors and cytokines in the neointima. Many reports have shown that PDGF, TGF-ß, EGF, bFGF and IL-6 can stimulate TIMP-1 synthesis [31,32,33]. The regulation of TIMP synthesis occurs predominantly at the transcriptional level, although some synthesis regulation at post-transcriptional level has also been described [34]. The transcriptional response of the TIMP-1 gene is regulated by multiple regulatory sequences lying within 1Kb upstream of the transcription start site, including binding sites for transcription factors, like activator protein 1 (AP-1) and Polyoma enhancer A binding factor 3 (PEA-3) [11]. These regulatory mediators act in consort for rapid transcription. Interestingly, many of these mediators are synthesized by neointimal SMCs and are uniformly distributed throughout the neointima [35]. In addition to the mediators, the mRNA stability, a post-transcriptional property, also takes part in TIMP-1 regulation. This post-transcriptional regulation might be responsible for the differences in the degree of elevation in TIMP-1 synthesis at mRNA and protein level in neointimal SMC [36].

Information is emerging about the role of TIMPs in various disease states. The elevation of TIMP is implicated in osteoarthritis [37] and tumour metastasis [38,39]. It has been suggested that tumour cell invasion, a consequence of repeated attachment of tumour cells to ECM and their subsequent release, is mediated by TIMP [40]. The elevated TIMP-1 synthesis in neointima raises some question about its role in atherogenesis, a process characterised by enhanced degradation of ECM by metalloproteinases. A number of reports have shown an increased expression of MMPs, particularly 92 KDa gelatinase and collagenase in atherosclerotic plaques and neointimal tissues [20,41]. If TIMP synthesis is increased to counter-balance the increase of MMP, then there might not be the accelerated degradation of ECM, which has been frequently seen in developing

atherosclerotic lesions as well as in the neointima covered by regenerated endothelium. One explanation could be that increase in TIMP, though parallel to the increase in MMP, is not enough for complete inactivation of MMP. Another possibility is that TIMP becomes more involved in functions other than inhibiting metalloproteinase. Hayakawa *et al.* have documented that TIMP promotes fibroblast and endothelial cell proliferation [42] and more recently, TIMP has also been implicated in cellular migration in tumours and in angiogenesis [38,43]. It is likely that TIMP stimulates SMC migration and proliferation, thereby facilitating atherogenesis.

5.6. Acknowledgements:

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CHAPTER 6

6.1. Overall conclusion

The word atherosclerosis derives from two Greek terms: athere, porridge, and sklerosis, a hardening. The porridge primarily refers to the lipoprotein deposition and the hardening represents the accumulation of ECM components. It is currently accepted that ECM actively take part in atherogenesis related to lipoprotein retention and SMC activity. However, metabolism of ECM in the atherosclerotic lesion is still not well understood.

The balloon catheter deendothelialization of rabbit aorta is a well-established model to study atherosclerosis. The neointima developed up to 15 weeks after arterial injury has many features of early atherosclerotic lesions. We and others have previously shown increased accumulation of ECM components, such as collagen and proteoglycan, in this lesion. However, it is known that both collagen and proteoglycan contain many subtypes which have different biological functions.

The first part of my study examined mRNA expression of certain ECM components: collagen type I, collagen type III, and syndecan-1. The expression of all measured mRNA is increased, in accordance with the overall up-regulation of ECM in atherosclerotic lesions. Detailed analysis revealed that the extent of increase is different: three fold for collagen type III, six fold for collagen type I. Syndecan-1 mRNA is not detectable in normal media, but its expression is prominent in the neointimal tissue. The enhanced synthesis of the ECM components tends to facilitate LP sequestration in the neointima, because all three ECM components have relatively high affinity for LDL. Collagen, especially type I and III collagen, takes part in platelet aggregation after arterial

injury. As an integral membrane PG, syndecan-1 can also mediate the interactions between ECM and the cytoskeletal system, and thus contribute to SMC activation.

Increased ECM content in the neointima constitutes a physical barrier for SMC migration to the intima. This migration is considered a primary feature of atherogenesis. Because of the potent activity of MMP in ECM degradation, their expression in the neointimal tissue was examined. Enhanced MMP expression was observed from the 4th week up to the 12th week and MMP positive cells are homogenously distributed in the neointima. It is concluded that synthesis of MMP after deendothelialization is a prolonged process and neointimal SMCs are an important source of MMP production. The tissue distribution of MMPs is in agreement with their active participation in SMC migration, as suggested by an in vitro study.

MMPs are potent proteinases and as a family, MMPs can digest all ECM components under physiological conditions. In human tissues, MMP activity is tightly regulated. For example, active MMPs can be inhibited by TIMPs. Studies were extended to evaluate the expression of a TIMP in the neointima. Data from these studies indicate that neointimal cells transcribe the TIMP-1 gene, the resultant mRNA has high homology with TIMP-1 mRNA from several other sources. Expression of this mRNA is significantly enhanced in the neointima. This enhancement continues from the 4th week to the 12th week after arterial injury. The tissue distribution of TIMP-1 is similar to that of MMPs. Increased TIMP-1 protein concentration in the neointima compared to normal media is also observed. The increased TIMP-1 expression reveals a mechanism to antagonize MMP activity in the neointima. The continuous enhancement of TIMP-1 expression indicates diminished ECM degradation with the continuing development of the neointima.

In summary, my studies indicate that alteration of ECM metabolism after arterial

deendothelialization is prominent and complicated. On the one hand, there is enhanced synthesis of ECM components. These components contribute to the occlusive nature of atherosclerosis and are involved in lipoprotein retention, SMC proliferation as well as platelet aggregation. In addition to the increased synthesis, breakdown of ECM may also occur via the proteolytic activity of MMPs. ECM degradation is a pre-requisite for SMC migration. However, this digestion is limited, as continuous augmentation of TIMP-1 expression is observed after de-endothelialization.

6.2. **Original contribution**

For the first time, this study showed the tissue distribution of syndecan-1 mRNA in the neointima, developed after de-endothelialization. Also for the first time prolonged existence of this mRNA in the neointima, i.e. 12 weeks after injury has been shown. These results set the stage for further analysis of this integral membrane PG in mediating extracellular signals to neointimal SMCs.

This study, for the first time, illustrated tissue distribution and continuous up-regulation of mRNA for MMP-2 and MMP-9 in the neointima. This provided strong support for a role of MMPs in atherogenesis, as previously suggested.

This study, for the first time, demonstrated the up-regulation of one MMP inhibitor, TIMP-1, in the neointima. This result showed that ECM degradation by MMP is a limited process, at least in the aortic neointima.

A rabbit TIMP-1 cDNA was isolated from neointimal tissue and sequenced. This cDNA was also expressed as a soluble TIMP-1 protein using a prokaryotic expression system. This provided powerful tools for further study.

Expression of mRNA for collagen type I and type III by medial and neointimal SMCs were compared using a cell culture system. Results of the study support the notion that enhanced synthesis contributes to ECM accumulation.