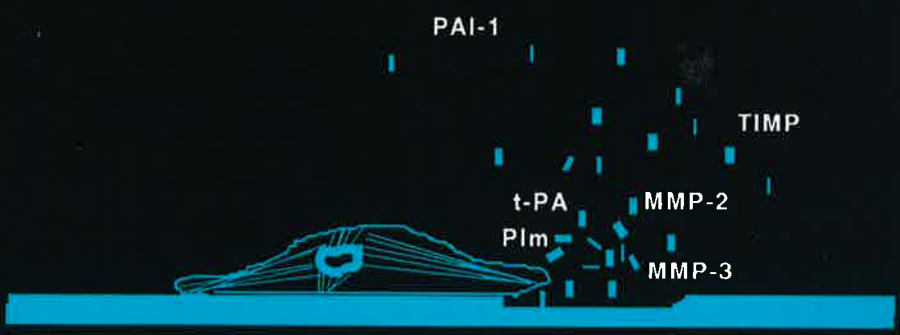
The Vascular Smooth Muscle Cell: Regulation of Extracellular Proteolysis.



René T. J. van Leeuwen

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STELLINGEN

Behorende bij het proefschrift

The Vascular Smooth Muscle Cell: Regulation of Extracellular Proteolysis.

1 Activatie van bloedplaatjes draagt niet alleen via het vrijkomen van chemotactische substanties bij aan het stimuleren van de migratie van gladde spiercellen door de vaatwand, maar ook via het stimulerend effect op de extracellulaire proteolyse van gladde spiercellen (*dit proefschrift*)

2 De beschreven reductie in thrombotische gebeurtenissen in patiënten herstellende van een myocard infarct, en van patiënten met een hoge-renine hypertensie die met ACE-remmers behandeld zijn, berust mogelijk op een door de verlaagde angiotensine II gemediëerde reductie in de productie van PAI-1 door vasculaire gladde spiercellen (*dit proefschrift*).

3 Gladde spiercellen zijn in staat extracellulaire matrix componenten af te breken via een plasmine-afhankelijke en een plasmine-onafhankelijke route (*dit proefschrift*).

4 Vele stimuli die de aanmaak van proteases in gladde spiercellen activeren, verhogen ook de aanmaak van hun remmers.

5 De meest gebruikte manier om gekweekte (proliferende) gladde spiercellen van fibroblasten te onderscheiden berust op het aankleuren met een antilichaam tegen α-(gladde spiercel) actine. Dit is echter geen juiste methode, gezien de waarnemingen van Darby *et al.* (*Lab Invest 1990; 63: 21-29*) dat myofibroblasten grote hoeveelheden α-(SMC) actine isovorm kunnen produceren, en van Pauly *et al* (*Circ Res 1994; 75: 41-54*), dat proliferende gladde spiercellen geen of weinig α-(SMC) actine aanmaken.

6 De waarneming van Bell en Madri (*Am J Pathol 1990; 137: 7-12*) dat angiotensine (Ang) -II in staat is, de migratie van gekweekte runder gladde spiercellen te stimuleren, maar Ang I niet, is in tegenspraak met de aanwezigheid van angiotensin-converting enzyme in gladde spiercellen.

7 Sasaguri *et al* (*Lab Invest 1994; 71: 261-269*) tonen met behulp van een chromogeen substraat "elastase activiteit" in vasculaire gladde spiercellen aan. Gezien de beperkte specificiteit van dergelijke substraten is deze conclusie op zijn minst voorbarig.

8 Vanwege de sterke afstoting van donorhuid, is een kunstmatig huid-equivalent de te prefereren oplossing om een functioneel en cosmetisch acceptabele permanente bedekking te verkrijgen bij diepe huidwonden.

9 De typische Hollandse zuinigheid om niets te willen weggooien, is er de oorzaak van dat men bij scheidingsprocedures van DNA of RNA een hoge opbrengst maar een lage zuiverheid verkrijgt.

10 In Italie leert men problemen uit te stellen in plaats van op te lossen met als argument dat als een probleem zich zelf niet oplost na enige tijd, het in het begin toch te gekompliceerd was om op te lossen.

11 Cellen uit de vaatwand kruipen waar ze niet gaan kunnen.

Leiden, 6 juni 1996.

René van Leeuwen

The Vascular Smooth Muscle Cell: Regulation of Extracellular Proteolysis.

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
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volgens besluit van het College van Dekanen
te verdedigen op donderdag 6 juni 1996
te klokke 15.15 uur

door

Reinier Theodorus Jacobus van Leeuwen

geboren te Lisse in 1961

"ALL INVERHAGEN BV" - KATWIJK

Promotiecommissie:

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CONTENTS

CHAPTER 1.

GENERAL INTRODUCTION

Contents

1.1 Pathological changes in the vessel wall.

Atherosclerosis is a disorder characterised by the presence of lesions or plaques in the intima of arteries. Atherosclerosis is a widespread phenomenon leading to several pathological consequences of which myocardial and cerebral infarctions are very frequent and life-threatening manifestations. Equally frequent, but more chronic consequences of atherosclerosis are chronic ischemic heart diseases (such as angina pectoris), peripheral vascular disorders (claudicatio intermittens) and multi-infarct dementia. The mechanisms leading to atherosclerotic lesions are not yet completely understood, but the clinical manifestations all arise from a compromised blood circulation that leads to ischemia. The lesions are often regarded as being a consequence of acute or chronic injury to the vessel wall, in particular the endothelium. In the developing atherosclerotic plaque inflammatory processes often take place, monocytes and macrophages infiltrate the lesion and in addition vascular smooth muscle cells, lipids and cholesterol accumulate. Eventually, the blood circulation may be impeded, and by that time clinical manifestations emerge. When such a plaque ruptures, a thrombus may result, that can cause an acute and total blockage of the circulation. Atherosclerotic plaques can be surgically removed and the circulation can be restored by percutaneous transluminal (coronary) angioplasty (PTCA) or alternatively the stenosis can be by-passed with a short stretch of (donor) vessel (coronary artery by-pass grafting or CABG). However, even if this intervention is initially successful, restenosis frequently occurs. Secondary stenosis (restenosis) poses a great problem in the management of vessel obstructions. Factors that contribute to this secondary stenosis are discussed below.

1.1.1 Morphology of atherosclerotic lesions.

Atherosclerosis is a disorder characterised by the presence of lesions or plaques in the intima of the medium-sized and larger arteries. The pathology of atherosclerosis has been recently reviewed. [1,2,3] Clinically, atherosclerotic lesions have been classified in three groups of increasing severity: [4,5,6]

Fatty streaks are small lesions: local areas of intimal hypertrophy with some incorporated smooth muscle cells and monocytes, that are loaded with lipids (foam cells). These intimal smooth muscle cells are of another phenotype than in the media and display a higher metabolic activity. In these small lesions, fibres of connective tissue may be found. Fatty streaks occur also in young and healthy people. There is no apparent connection with any risk of cardiovascular disease. But they often occur in the same locations as where mature atherosclerotic lesions are found at a later age, and may be the precursors of atherosclerotic lesions. [5,7]

Atherosclerotic or fibrous plaque. These plaques have a typical histological appearance, and are recognised macroscopically as a white, elevated area on the intima of the vessel

wall. The intimal thickening consists of a core, sometimes called an atheroma, containing extracellular lipids, cholesterol, calcium salts, and necrotic cell debris. The atherosclerotic plaque is covered with a fibrous connective tissue cap. Monocytes and smooth muscle cells are found abundantly and may develop into foam cells.
Complicated lesion. Calcification and fibrosis of the lesion is observed, accompanied by necrosis and ulceration around the lesion, thrombosis on the surface and further downstream. Bleeding often occurs inside the complicated lesion, and rupture of the fibrous cap which may lead to the formation of mural thrombi that can completely obstruct the lumen of the vessel. An acute myocardial infarct is often caused by thrombosis as a consequence of a ruptured coronary plaque.

1.1.2 Atherosclerosis and primary stenosis.
Ross [5,7] has formulated the "Response to injury hypothesis" based on clinical observations and animal studies. He distinguishes (1) denuding and (2) non-denuding injury.
-1- Denuding injury. In the case of extensive endothelial damage, the prothrombotic subendothelium becomes exposed to the circulating blood. Tissue factor (TF), fibronectin and collagen in the subendothelium promote platelet activation and thrombus formation (denuding injury). Platelets secrete high local concentrations of growth-factors, such as PDGF, TGF-ß and EGF. Following this sequence of events, the underlying medial smooth muscle cells can start to proliferate and to migrate. [2,5] A fibromuscular plaque, or neointima is formed. The platelet factors PDGF, platelet factor (PF)-4, ß-thromboglobulin and prostaglandins have a strong chemotactic effect on circulating monocytes. In addition, smooth muscle cells in the neointima are able to secrete JE (monocyte specific chemotactic factor) and KC (neutrophil specific chemotactic factor). [8] As a consequence monocytes migrate into this fibromuscular plaque and to develop into macrophages and foam cells that deposit lipids (including cholesterol and LDL) in the lesion. Endothelial cells can oxidise the incorporated lipids, and oxidised LDL especially can induce monocyte chemotaxis and activation, [7] and contribute to the local damage, cell death, necrosis, and thrombosis. SMC proliferation usually stops when endothelial integrity and functionality has been restored, but the detrimental effect of oxidised lipids can continue for a long time. The administration of anti-oxidants can diminish experimental atherosclerosis in some cases. [1] The processes causing intimal thickening may eventually lead to a partial or even total obstruction of blood flow in the vessel. Often however the atherosclerotic plaque ruptures and acutely gives rise to a mural or an occluding thrombus.
-2- Non-denuding injury. Even if the rate of proliferation of endothelial cells at the site of damage is high enough to maintain a continuous endothelial layer, mural thrombi may still arise, because the activated, proliferating endothelial cells are less anti-thrombogenic than quiescent endothelial cells. These activated endothelial cells can

produce a host of growth factors that can activate the underlying smooth muscle cells, including PDGF, bFGF, TNFα, IL-1, and M-CSF. Evidence has accumulated that oxidised LDL is a key component in the initial activation or injury of the endothelium, after this initial activation of endothelium, a cascade of reactions such as proliferation of smooth muscle cells and incorporation of macrophages and lipids into the intima may take place as mentioned in 'denuding injury'. This non-denuding type of damage is reported in patients with high LDL levels, with diabetes, or hypertension, and in smokers. It is now seen as a more probable and common way to develop atherosclerosis than via complete denudation of the endothelium. [7]

1.1.3 Restenosis after PTCA.

Stenosis of the arteries can be treated by surgically removing the atherosclerotic plaque or by angioplasty. An important drawback of the success rate of intervention in CVD is the occurrence of restenosis of the vessel after the operation. Restenosis after percutaneous transluminal coronary angioplasty (PTCA) occurs in a large percentage ($\sim$30%) of the cases treated. [9] A number of treatments that inhibit restenosis in animal models, unfortunately not always effective in the clinic, have been described; such as with anti-platelet agents (aspirin, dipyridamole, theophyllin), heparin, ACE-inhibitors (Captopril, Lisinopril), anti-inflammatory agents (corticosteroids), fish-oil rich diets (reviewed in [10,11,12]).

Casscells [13] has formulated a model of restenosis after PTCA based on data obtained using the rat carotic artery denudation model. He distinguishes between two separate phases:

-1- Early stenosis (between 1 hour and 2 days after PTCA) which involves mainly thrombus formation, and vasospasm caused by vasoactive substances that are released after the intervention. This phase is reversible.

-2- Late stenosis (weeks after PTCA) which involves smooth muscle cell proliferation, intimal hypertrophy and other, essentially irreversible changes in the vessel wall. It leads to a dense collagen scar, containing smooth muscle cells, collagen and proteoglycans, but few inflammatory cells and hardly any lipids. The secondary lesion may eventually cause a complete obstruction of the vessel. Restenosis is less inflammatory in nature than the primary atherosclerosis. [13,14,15]

1.1.4 Experimental models

A lot of the knowledge of the progression of (1) atherosclerosis and (2) secondary stenosis is obtained from experimental animal models.

Ad 1- Emphasis was initially put on the relation between serum cholesterol levels and the degree of atherosclerosis. A high cholesterol diet in non-human primates [16] induces lipid-rich lesions that resemble the human "fatty streak" lesions. In this and other

models a migration of medial smooth muscle cells towards the intimal lesions and a subsequent proliferation of these intimal SMC was observed, together with an accumulation of macrophages. Many model systems for vessel injury and atherosclerosis make use of high-cholesterol or -triglyceride diets to induce the damaging lipid accumulation within the vessel wall. Several model systems of dietary-induced arterial injury have been reviewed. [5] It must be noted that these models all describe the atherosclerosis that develops in essentially healthy animals on a very unnatural diet. The aetiology of this type of atherosclerosis is very different from that in animals and humans on a regular diet, but with a pre-existing disorder such as familial hypercholesterolaemia. It is possible to study atherosclerosis in animals with a pathological metabolism on a regular diet. The Watanabe heritable hyperlipidemic (WHHL) rabbits which lack a functional LDL-receptor and are characterised by elevated serum LDL-levels and rampant atherosclerosis. [17,18]

Ad 2- Other models put more emphasis on mechanical injury as a cause of atherosclerosis. These models are probably better suited to mimicking secondary stenosis.

- Baumgartner described the catheter-induced abdominal aorta injury model in the rabbit as early as 1963. This damage caused adhesion of platelets and monocytes to the wounded area. [19]
- Fishman caused vessel damage by air drying the intima in the rat carotid artery, a procedure that avoids stretch or other significant damage to the underlying media. A striking myo-intimal proliferation was observed in the damaged stretch of the vessel. [20]
- van Aken caused a thrombus in the rat aorta by inverting a small flap of vessel wall tissue. This was followed by migration of smooth muscle cells from this flap into the surrounding thrombus. [21]
- Clowes [22] started using the rat carotic artery "balloon injury model", which still is the most widely used model of vessel wall repair: the endothelium in one of the carotid arteries is rubbed off with a balloon catheter and the events in the injured vessel wall are compared with the untreated one.
- Another well recognised model is the rabbit carotic artery "perivascular manipulation model" first published by Booth *et al.* [23] : mechanical stress and/or hypoxia induce damage under an impermeable cuff while the endothelial layer remains continuous. This model demonstrated that removal or direct damage of the endothelium is not necessary to induce SMC proliferation.

In addition to these animal models, cultured vascular smooth muscle and endothelial cells have significantly expanded the knowledge of processes that are involved in cell migration and proliferation in connection with atherosclerosis and restenosis.

Based on the rat carotic artery model Clowes distinguished four 'waves' of migration

and proliferation after arterial endothelium injury. [22,24]
-The first wave between 1 and 3 days after injury is characterised by DNA synthesis in medial smooth muscle cells.
-The second wave between day 3 and 4 is characterised by migration of smooth muscle cells from the media towards the intima.
-The third wave takes place between 4 and 8 days, but continues up to 12 weeks, and includes proliferation of the migrated smooth muscle cells and formation of a fibromuscular neointima, rich in smooth muscle cells, [25] and extracellular matrix components that are synthesised by these neointimal SMC. [26]
-During the fourth wave or re-stimulation phase the neointimal smooth muscle cells are hyper-responsive to several growth factors, whereas the quiescent medial SMC are relatively refractory to many responses. [27,28,29]
Simultaneously with the SMC migration and proliferation, the damaged endothelium starts to proliferate and to migrate inward from the edges of the denuded area. This process proceeds by about 2 mm per week until it stops after about 6-10 weeks. If the denuded area is very large, the central region will never be re-endothelialised. [30,31]

1.2 The vascular smooth muscle cell in its environment: the vessel wall.

The vessel wall consists of three layers: the *intima*, a layer of endothelial cells, the *tunica media* and the outer *adventitia*. The tunica media forms the greatest mass of the larger arteries and consists solely of fibrous extracellular matrix material and smooth muscle cells. [32] The smooth muscle cells (SMC) are usually arranged in one concentric and one longitudinal layer in an interstitial matrix of elastin and collagen fibres. The matrix in the media consists 50% of elastin, [33] and 30% of collagen, predominantly types I, type III, and type V; [34] the rest is mainly fibronectin, thrombospondin and (other) glycoproteins. [26,35,36] The boundary between the adventitia and the media is the *lamina elastica externa,* consisting of elastin and collagen. The media is separated from the intimal layer of the vessel by the *lamina elastica interna*, containing laminin, collagen type-IV, heparansulphate-proteoglycan and entactin (or nidogen). [35]

1.2.1 Smooth muscle cell phenotypes

The vascular smooth muscle cells can appear in at least two forms or phenotypes with different mechanical and biochemical properties: Chamley *et al.* distinguished between the contractile and the synthetic phenotype. [37,38]
-The cells displaying a *contractile phenotype* are in a quiescent, non-mitotic state, have a well developed contractile apparatus, and their main function is mechanical: to maintain vessel wall rigidity, elasticity and blood pressure. These are the normal resting

cells in the media of healthy adult arteries. In this state they do not possess the PDGF-receptor and are refractory to PDGF stimulation. [39] Their actin is entirely of the α-SMC-actin sub-type. Contractile smooth muscle cells respond to angiotensin II (10^{-7} g/l) by a 60-80% contraction of length. [38]

-The cells of the *synthetic phenotype* are in a mitotic state and have a less well-developed actin-myosin system. [40,6] After switching from contractile to synthetic phenotype, the total volume of myofilaments in SMC decreases from 54 to 20 % and a considerable amount of ß-actin is present in addition to the α-SMC form. The amount of α-actin mRNA is reduced. [41] They do not contract after the addition of angiotensin II. Synthetic smooth muscle cells have more rough endoplasmatic reticulum and a larger Golgi-apparatus. This is the cell type found in the neointima, but also when medial SMC are cultured *in vitro* they assume this phenotype after a few generations. Synthetic SMC produce autocrine cytokines such as PDGF, IGF-1, IL-1, and TNF-α and the synthesis of extracellular matrix proteins is enhanced. [34] Synthetic type SMC express the PDGF-receptor and are susceptible to growth stimulation by whole blood serum and PDGF. [38,6] The lipoprotein receptor is increased and therefore normolipidemic LDL and particularly hyperlipemic LDL are mitogenic for synthetic smooth muscle cells, but not particularly so for contractile SMC. [38]

After injury or stress the contractile cells can develop into the synthetic type. The transition between these two phenotypes is reviewed. [42] It is not clear whether all cells have this potential to switch phenotype, or if a dormant sub-population of pre-synthetic cells is present within the vessel wall. [6] The differences between the quiescent medial smooth muscle cells and the synthetic neointimal SMC seem to be quite substantial and (semi-) irreversible: rat vascular SMC cultured from the media and neointima 2 weeks after carotic artery de-endothelialisation maintain a different morphology after several generations in culture. The intimal SMC in culture have a more epithelium-like phenotype than medial SMC, and produce more platelet-derived growth factor (PDGF) than their counterparts. [43]

Cell-cell contacts can inhibit the phenotype transition: if the seeded primary medial smooth muscle cells are seeded in high densities they do not change their phenotype, if they are seeded in lower densities but reach confluence within 5 cell divisions they revert to the contractile phenotype, in other cases they revert to the synthetic state. [40]

1.2.2 Interaction between smooth muscle and endothelial cells.

Endothelial cells and smooth muscle cells influence each other's metabolism. The endothelium is a source of molecules that can regulate the proliferation of smooth muscle cells. [44] The influence of damaged endothelium on SMC metabolism is reviewed, [45] as well as their interaction in the development of hypertension. [46]

Confluent endothelial cells in a co-culture system [47] or conditioned medium from confluent endothelial cells [6] can keep SMC in the contractile state. Quiescent endothelial cells synthesise certain molecules, such as heparansulphate-proteoglycans and TGF-β that inhibit SMC growth and migration. [13] Proliferating smooth muscle cells on the other hand, inhibit the growth of endothelial cells. [13] Co-culturing endothelial cells and smooth muscle cells on either side of a porous membrane results in the inhibition of endothelial cell proliferation. The arrangement of the cells in this co-culture system mimics the *in vivo* orientation of vascular cells in which medial smooth muscle cells are separated from the abluminal surface of the endothelium by a fenestrated internal elastic lamina or basement membrane. [48] Proliferating endothelial cells produce bFGF which is a strong mitogen for SMC. When the endothelial layer is restored, the endothelial cells revert to the resting state and stop secreting bFGF. [49,50,51,52]

1.2.3 The extracellular matrix.

The smooth muscle cells are embedded in a layer of elastic and fibrous material secreted by SMC. In the lamina elastica externa this material is very fibrous, rich in collagen and elastin bundles, and provides the strength and elasticity of the blood vessel. SMC excrete varying amounts of components of the ECM, dependent on their state of activation: collagen type-I, -III, -V, microfibrils, fibronectin, elastin, glycosaminoglycans cross-linked with heparansulphate, dermatansulphate, chondroitinsulphate and hyaluronic acid.[36,53,34,33,54,55]

The ECM not only serves as supporting and filling material between the cells, providing rigidity and elasticity to the blood vessel, but it is an important bi-directional medium for communication between the cells. Confluent, quiescent endothelium and smooth muscle cells secrete heparansulphates, but also laminin and collagen type-IV in the ECM. These matrix constituents are important factors regulating the phenotype of cultured SMC; [56] laminin and collagen type-IV, [56] or 'Matrigel' (mainly collagen IV and laminin), heparin [57,58] and heparansulphates [59] have been reported to sustain the contractile appearance of seeded primary vascular smooth muscle cells in culture, whereas they assume the synthetic phenotype on non-treated culture plastic or on plastic with a fibronectin, [60] collagen type-I or type-III (40) coating. In contrast cells seeded *within* a three-dimensional matrix of collagen-I maintained their contractile phenotype. [40,61] When the endothelium is damaged the ECM proteins become exposed to the bloodstream. Some ECM components are procoagulant (such as PAI-1, fibronectin, factor-V, van Willebrandt factor-factor VIII, collagen, tissue factor), others are anticoagulant (heparin, heparansulphates and thrombomodulin). The exposed ECM is an anchor for circulating platelets and monocytes. [62] They can cause high local concentrations of growth factors and chemoattractants. These platelet and monocyte products can lead to the proliferation of smooth muscle cells and endothelium, and to the migration of SMC and influx of circulating leukocytes. [5,2]

1.2.4 The regulation of migration and proliferation in smooth muscle cells.

Intimal damage is followed by both migration and proliferation of medial smooth muscle cells. These processes are often coupled, but they are different mechanisms under control of different signals. Migration is not dependent on mitosis; it still takes place after DNA synthesis is blocked. [13,63] According to Clowes *et al.* the majority of migrating smooth muscle cells will never divide, [25,13] but a few clones of migrated cells are responsible for the neointimal smooth muscle proliferation. [4,59,64]

Many triggers have been proposed as the cause of the onset of both migration and proliferation. According to Ross [5] denuding or non-denuding damage of the endothelium causes activation of platelets, local release of platelet factors, followed by SMC migration and proliferation. The platelets products are not sufficient to explain the SMC proliferation, as anti-coagulants, platelet inhibitors, and anti-platelet antibodies only moderately inhibit smooth muscle hyperplasia. [65] On the other hand, SMC migration from the media to the intima after balloon denudation is strongly inhibited in animals with severe thrombocytopenia. [13] These findings suggest that SMC proliferation after intimal injury is largely independent of platelet products, and that platelet factors are mainly chemoattractants for medial SMC. The mechanisms that are involved in the onset of migration and of phenotype switch and proliferation of medial smooth muscle cells are discussed in more detail below.

1.3 Migration of smooth muscle cells

During the development of both primary atherosclerotic lesions and restenosis, smooth muscle cells emerge in the intima. These SMC originate from the arterial media. In the rat carotid artery model, migration of smooth muscle cells from the media to the intima takes place 24-72 hours after vessel wall damage. [24] Migrating smooth muscle cells have to detach themselves from their surrounding extracellular matrix and to degrade extracellular matrix material in order to be able to move through the vessel wall. Extracellular proteolysis in the immediate vicinity of these migrating SMC is increased in order to enable this local matrix degradation. Co-ordinate and consecutive activation of plasminogen activators, plasmin and metalloproteinases is reported during the migration of smooth muscle cells. [66,67,68] Recent progress on the knowledge of the regulation of extracellular proteolysis in the migrating smooth muscle cell and the implications for stenotic vascular disorders is discussed in the second chapter of this thesis.

1.4 Hyperplasia of vascular smooth muscle cells

In atherosclerotic lesions, but also in the secondary lesions that appear after treatment of primary stenosis, intimal hypertrophy is observed. This hypertrophy is characterised partly by increased production of extracellular matrix proteins and partly by an increase in the number (hyperplasia) and volume (hypertrophy) of cells, especially smooth muscle cells in the afflicted area. A sub-population of the quiescent, contractile medial smooth muscle cells changes into proliferating, intimal smooth muscle cells in a synthetic state.
Many cytokines and growth-factors are known to modulate both the number of smooth muscle cells and the excretion of ECM proteins. There is an extensive trafficking of signalling molecules between smooth muscle cells and endothelial cells, monocytes/macrophages and platelets that are present in or around the vessel wall. Factors modulating the proliferation and the transition to the synthetic state of smooth muscle cells are summarised below and schematically represented in figure 1.

1.4.1 Mechanical factors

In addition to growth factors, mechanical factors too stimulate the vascular smooth muscle cells. This was supported by the finding that balloon catheterisation under high pressure, causing injury deep in the vessel wall with little thrombosis has more effect on SMC proliferation than superficial injury with massive and occlusive thrombosis. [13] The mechanical forces during PTCA are very strong. Stretch-inducible Ca-channels are reported to exist in SMC and they stimulate the contractile apparatus of medial SMC. [69] Stretch increases the intracellular Ca^{2+} and inositol-triphosphate levels, and the subsequent induction of proto-oncogenes may be one of the first steps in the phenotype switch and mitosis. [13,70,71] Ca-channel blockers like nifedipine can inhibit SMC proliferation in a rat and rabbit catheter injury model when they are administrated at the time of injury or within the first day. [72,73,74] The Ca^{2+} antagonist verapamil (and diltiazem and nifedepine as well) inhibited PDGF-induced proliferation of cultured rat aortic SMC [74,75] but not the proliferation induced by pulsatile stretch. [75] From the observation that verapamil does not inhibit these stretch-induced Ca-channels we conclude that they are different from the PDGF-receptor induced Ca-channels, and we predict that the efficacy of Ca-channel blockers to reduce intimal proliferation after PTCA would be enhanced if both types of Ca-channels were blocked. Specific blockers of stretch-induced Ca-channels do however exist, among them is Gadolinium, [76] but these blockers have not yet been tested on their ability to inhibit SMC proliferation after vessel injury.

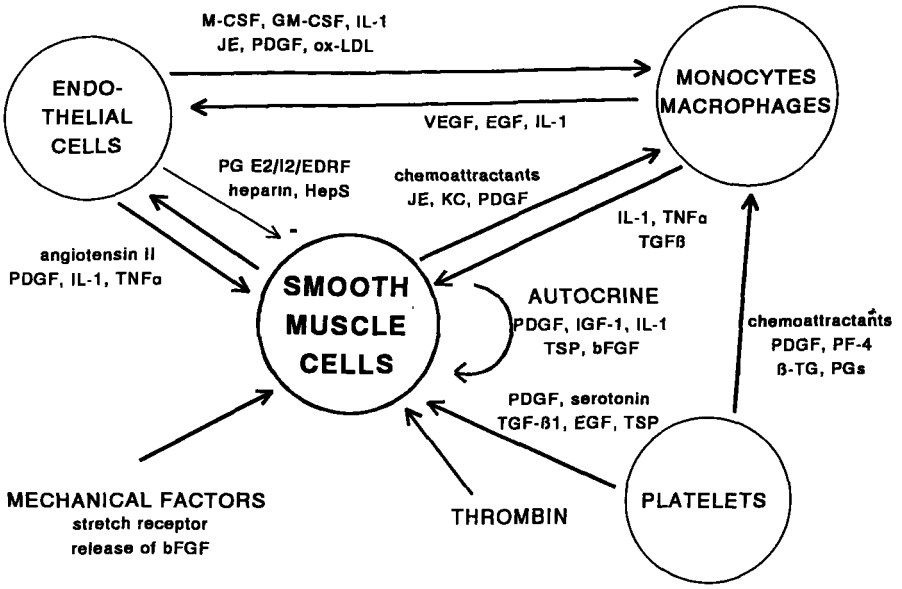

FIGURE 1

Summary of the interactions between smooth muscle cells and factors from other cells within the vessel wall, or from the blood, modulating the phenotypic state of the vascular smooth muscle cell. Platelet products and thrombin can activate the smooth muscle cell (SMC). Endothelial cells can either activate or inhibit (thin arrow) the SMC, macrophages and monocytes are attracted by chemoattractants from activated platelets or SMC and can further activate the SMC. The interactions involved in smooth muscle cell activations are very complex and dynamic. bFGF=basic Fibroblast Growth Factor; EDRF=Endothelium Derived Relaxing Factor or nitric oxide; EGF=Epithelium Growth Factor; (G)M-CSF=(granulocyte-) monocyte colony stimulating factor; HepS=Heparansulphate proteoglycans; IGF-1=Insulin-like Growth Factor-1; IL-1=Interleukin-1; JE=monocyte specific chemotactic factor; KC=neutrophil specific chemotactic factor; ox-LDL=oxidised Low Density Lipoprotein; PDGF=Platelet Derived Growth Factor; PF4=Platelet Factor 4; PGs=Prostaglandins; β-TG=Beta-thromboglobulin; TGF-β=Transforming Growth Factor-beta; TNF α/β=Tumor Necrosis Factor α or β; TSP=thrombospondin; VEGF=vascular endothelial growth factor.

1.4.2 Growth factors

The growth factors that modulate smooth muscle cell metabolism can be divided in several groups according to their origin *in vivo*:

-1- Factors from platelets

When the endothelium is damaged, platelet activation and thrombus formation may occur. The release of the content of these platelets causes high local concentrations of growth-factors, the most important being PDGF, serotonin (5-HT), TGF-ß and EGF.

-*PDGF* is responsible for about 50% of the growth stimulating activity of platelet lysate on cultured smooth muscle cells. [77] *In vivo* PDGF is a potent chemoattractant rather than a mitogen for the contractile medial SMC. [78,65,79,80,51] In contrast PDGF is one of the strongest known mitogens for the synthetic phenotype *in vivo* and the cultured

vascular SMC. [39,81,82]

-*Serotonin* or 5-HT is known to stimulate *in vitro* smooth muscle cell proliferation of porcine, bovine, rabbit and human origin, [83,84,85,86,87] and therefore may be linked to the development of intimal hyperplasia.

-*EGF*. Epithelium growth factor (EGF) induces thymidine incorporation in porcine arterial smooth muscle cells, but is not as potent as PDGF or FCS.[83]

-*TGF-ß1* is a growth factor with opposite effects on SMC proliferation depending on the concentration. TGF-ß has a bimodal effect on human neonatal aortic SMC; it increases ^{3}H-thymidine incorporation at low doses (between 1-10 fg/cell), but above 10 fg/cell thymidine incorporation decreases. [88] TGF-ß1 increases thymidine incorporation in vascular smooth muscle cells isolated from the spontaneously hypertensive rat approximately twofold without a corresponding increase in cell number. [89] TGF-ß1 decreases the mitogenic effect of EGF. [88]

-2- Growth factors from mononuclear leukocytes

In the thrombi that are often found near intimal lesions, activated platelets excrete not only PDGF, but also platelet factor (PF)-4, ß-thromboglobulin and prostaglandins that have a strong chemotactic effect on monocytes. In addition, the activated smooth muscle cells in the neointima are able to secrete JE (monocyte specific chemotactic factor) and KC (neutrophil specific chemotactic factor); this attracts high numbers of mononuclear cells. [8] Monocytes and macrophages can invade the atherosclerotic lesions and produce the growth factors PDGF-BB, IL-1ß, TGF-ß1, TGF-α, bFGF, and EGF but inhibitors of inflammation such as steroids, non-steroid anti-inflammatory drugs (NSAID) and cyclosporin have not been very effective in preventing neointimal proliferation. [13] This suggests that inflammation is not the most important factor determining neointimal smooth muscle proliferation.

-3- Intracellular growth factors from damaged tissue

The damage that is inflicted on SMC, endothelial cells and their surrounding extracellular matrix during catheterisation or PTCA causes the release of internal mitogens, in particular basic fibroblast growth factor. [50,90] The release of basic fibroblast growth factor (bFGF) from damaged endothelium and smooth muscle cells [91,92,93,52] is an important factor contributing to the switch from the quiescent to the synthetic phenotype. In isolated aorta rings, basic FGF increases ^{3}H-thymidine incorporation after 48-72 h with 60%. [94] bFGF stimulates proliferation in cultured SMC, [50] but also medial SMC *in vivo*. [93] Dividing and migrating (injured state) endothelial cells after balloon catheterisation produce bFGF, a strong mitogen for SMC, [49] in addition bFGF is released from ECM and also from intracellular depots. [90] Inhibiting antibodies against bFGF inhibit up to 80% of the thymidine incorporation that is observed in medial SMC *in vivo* between 1-2 days after intimal injury. [50,29,91]

-4- Factors from the coagulation cascade.

The procoagulant thrombin is found near damaged endothelium after platelet activation, in the mural thrombi and complicated lesions, and after plaque rupture. In addition to its prothrombotic properties, it also has growth potentiating activity. Messenger RNA for the thrombin-receptor has been identified in human atherosclerotic lesions. [95] Thrombin increases ^{3}H-thymidine incorporation in cultured human arterial smooth muscle cells; its antagonist hirudin abolishes the thrombin effect. [96] This effect was also reported in cultured rat aortic SMC.[97]

-5- Other growth factors.

Ang II is a vasoactive substance with a marked effect on the metabolism of smooth muscle cells too. It is still controversial whether angiotensin (Ang) II has direct mitogenic effects on smooth muscle cells [98] or merely increases hypertrophy and protein synthesis. [99,83,100] This can be partially explained by the fact that angiotensin has both mitogenic effects and induces the synthesis of anti-proliferative substances in the SMC, such as prostaglandins, that will be discussed below.

IL-1 can stimulate the switch to synthetic phenotype by inducing autocrine interleukin-1 and PDGF-A synthesis. Interleukin-1, like angiotensin II, has both mitogenic and anti-proliferative effects on the smooth muscle cell. The prostaglandins PGE1 and PGE2, potent autocrine growth inhibitors of SMC, are induced by IL-1. [101]

IGF. Insulin-like growth factor 1 (IGF-1) is known to be mitogenic for vascular smooth muscle cells. [102,103] IGF-1 has been proposed as one of the mediators of vascular change because of its ability to stimulate proliferation in cultured vascular smooth muscle cells. [104] IGF-1 is an autocrine growth factor; mRNA transcripts in cultured rat aortic smooth muscle cells were markedly increased by PDGF AB or BB dimers. [105] Aortic IGF-1 mRNA increased several fold after balloon denudation with a peak at 7 days after injury. [106]

Many growth factors have a long-term effect on smooth muscle cells, and are in fact autocrine growth factors; they can stimulate their own synthesis or the synthesis of other autocrine growth factors, which prolongs the time that a growth factor is active. But they can also cause long-lasting changes in the extracellular matrix surrounding the SMC that prolongs the mitogenic state of the stimulated SMC. PDGF, TGF-ß and Ang II induce the thrombospondin (TSP) gene strongly and rapidly. TSP is a matrix protein that is essential for SMC growth. It has EGF-domains and binds to integrins present on the SMC. The turnover of TSP in matrix is quite slow, which causes the semi-irreversible growth promoting effect that some growth factors display. TSP keeps the VSM for a long time in an activated, dedifferentiated state. [107,108] TSP is also excreted by platelets and incorporated into thrombi. [109] A close contact between thrombi and intimal smooth muscle cells, as in intramural thrombi, may enhance the growth promoting effect of platelet factors released by these thrombi.

Factors that inhibit SMC proliferation are also known.

Heparin. Heparin inhibits baboon smooth muscle cell proliferation. [57] Heparin decreased the rate of DNA synthesis stimulated by PDGF in synchronised primary cultured rat aortic smooth muscle cells. [110] Heparin prevents the human arterial smooth muscle cell from entering the S phase of the cell cycle by binding and inactivating serum mitogens such as PDGF. [111] The growth inhibiting effect of heparin can be partially attributed to its ability to interfere with the thrombospondin (TSP)-integrin contact. TSP is essential for SMC growth. [107,108]

EDRF or nitric-oxide. In rat vascular smooth muscle, the endothelium-derived relaxing factor (EDRF) inhibits proliferation and migration, particularly that induced by angiotensin II. [69] SMC are not totally dependent on endothelium as a source of NO. In cultured arterial smooth muscle cells NO synthase activity is induced by LPS and IL-1. [112,113,114]

Prostaglandins. The prostaglandins PGE1 and PGE2 are potent inhibitors of SMC proliferation. [101] Some growth factors are potentially mitogenic for smooth muscle cells, but since they simultaneously promote prostaglandin production, the mitogenic effect is suppressed. Cultured RASM have both PDGF-α and PDGF-ß receptors. [81,100] Particularly PDGF-AA is able to stimulate the formation of arachidonic acid, PGE2 and PGI2 (prostacyclin), the prostaglandins suppress proliferation. [39] Angiotensin II increases the prostaglandin production in cultured rat aortic SMC. The cyclo-oxygenase inhibitor indomethacin completely blocks PGI2 production and markedly enhances the mitogenic effect of angiotensin. [115] This also applies to the interleukins IL-1α and IL-1ß that do not increase thymidine incorporation in quiescent cultured human, bovine or rabbit SMC when given alone, but when given together with the cyclooxigenase inhibitor indomethacin (1 μg/ml), both interleukins increase the thymidine incorporation 2-3 fold. [101]

In conclusion: Damage to vessel wall causes a reaction in the medial smooth muscle cells that involves a phenotype switch followed by migration towards the intima and myo-intimal proliferation. The signal for the phenotype switch and subsequent smooth muscle cell proliferation is complex: mechanical pressure or stretch, loss of integrity or viability of the endothelium, the presence of thrombin, activated platelets, oxidised LDL cholesterol and the release of growth-factors from the damaged tissue have all been mentioned. Possibly a combination of the factors mentioned is the most effective signal for the smooth muscle cell to switch phenotype and enter mitosis. The presence of intact and viable endothelium with its production of the inhibitory NO and heparansulphates is a factor that prevents SMC proliferation.

There is a balance between growth and growth inhibition of medial smooth muscle cells, and understanding this balance is important for developing therapies to treat or to prevent atherosclerosis and restenosis.

1.5 Outline and aims of the study

Migration of smooth muscle cells towards the damaged intima and the subsequent proliferation within the intima are key processes in the development of both atherosclerosis and secondary stenosis. The subject of this thesis is clarifying mechanisms that are involved in the process of migration of vascular smooth muscle cells, and in particular the balance between activation and inhibition of proteolysis in the vicinity of the migrating smooth muscle cell. The first two chapters give an overview of what is known in this respect: the role of the smooth muscle cell in relation to atherosclerosis and the relation between extracellular proteolysis and the migration of smooth muscle cells.

Plasminogen activators are able to increase cell migration. This has been demonstrated in tumour cells and in several non-transformed cell types. Inhibitors of these plasminogen activators are therefore important in the regulation of cell migration. Endothelial cells are able to synthesise large amounts of plasminogen activator inhibitor type-1 (PAI-1) after certain stimuli such as the administration of bacterial lipopolysaccharides (endotoxin). In chapter three endotoxin is used as an experimental stimulus. The production of plasminogen activator inhibitor type-1, both in cultured rat aortic smooth muscle cells, and *in vivo* in the aortic media of rats and rabbits was demonstrated.

There is ample evidence that platelet products stimulate the migration of medial smooth muscle cells towards the arterial intima after vessel wall injury. In the process of cell migration extracellular proteases are required. Two important classes of proteolytic enzymes are the plasminogen activators and the metalloproteinases. Platelet products such as platelet-derived growth factor (PDGF) are known to be chemotactic for vascular smooth muscle cells, but it was not known to what extent and how platelet activation contributes to the local proteolytic balance in the vicinity of vascular smooth muscle cells. In chapter four the effects were characterised that platelet products have on the production of plasminogen activators, metalloproteinases and their inhibitors by cultured rat aortic smooth muscle cells.

Hypertension has since long been associated with an increased risk of cardiovascular diseases. Angiotensin II is a vasoconstrictor and can induce hypertension. In addition this hormone has some other actions on vascular smooth muscle cells, including the induction of smooth muscle hypertrophy and proliferation. PDGF is another vasoconstrictor, a mitogen for SMC, and in addition it modulates the plasminogen activation and inhibition balance in cultured vascular smooth muscle cells. The local plasminogen activator activity is an important factor determining the ability of smooth muscle cells to migrate through the vessel wall. Angiotensin II shares with PDGF several steps in its signalling pathway and the question has arisen of whether Ang II is able to modulate the plasminogen activation in cultured rat aortic SMC. This question

was addressed in chapter five.

Two different pathways of proteolysis, involving plasminogen activators and metalloproteinases are thought to be involved in the migration of smooth muscle cells through the extracellular matrix surrounding migrating SMC. A technique was developed that enables the demonstration of two different pathways of matrix degradation. In chapter six a comparison was made between the degradation of extracellular matrix proteins by smooth muscle cells via a plasmin-dependent mechanism, and via a plasmin independent pathway, showing that both pathways of matrix degradation occur simultaneously.

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CHAPTER 2.

EXTRACELLULAR PROTEOLYSIS AND THE MIGRATING VASCULAR SMOOTH MUSCLE CELL.

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ABSTRACT.

Smooth muscle cells (SMC) form the major cell type in the arterial blood vessels. In the undamaged vessel wall they remain in a contractile state characterised by the absence of cell division, a low metabolic activity and a high actin-myosin content. As a reaction to injury of the vessel wall they can, however, change their phenotype and start to proliferate and migrate through the vessel wall from the media towards the intima. Extracellular proteolysis is increased during the migration of smooth muscle cells and local lysis of the surrounding extracellular matrix is observed. The smooth muscle cell produces several proteases during migration, especially plasminogen activators and metalloproteinases. Many growth factors and cytokines can modulate the production and activation of extracellular proteinases and their inhibitors in the activated vascular smooth muscle cell. The production and regulation of the extracellular proteolytic enzymes by smooth muscle cells in relation to cell migration are discussed in this review.

INTRODUCTION

Smooth muscle cells (SMC) form the most abundant cell type of the larger vessels. They can appear in two forms or phenotypes: the contractile and the synthetic phenotype. [1,2] In the normal, undamaged vessel wall, smooth muscle cells are in a contractile state characterised by absence of cell division, possession of a well-developed contractile apparatus and contraction after stimulation with vasoconstrictors such as angiotensin or serotonin. The main function of contractile smooth muscle cells is mechanical: maintaining vessel wall rigidity and elasticity and controlling blood pressure. As a reaction to stress or injury of the vessel wall they can, however, change their phenotype and start to proliferate and migrate through the vessel wall from within the media towards the intima. [3] These migrating cells assume the synthetic phenotype, characterised by, amongst others, increased production of extracellular proteinases and autocrine growth factors, while the total volume of myofilaments is decreased.
Several factors have been proposed in order to explain the switch in smooth muscle cell phenotype:
-Stretch and pressure forces in the vessel wall can activate stretch-inducable Ca-channels and activate medial smooth muscle cells. [4,5,6] The subsequent induction of proto-oncogenes may be one of the first steps in the phenotypic switch. [7]
-Changes in the interaction with the extracellular matrix in which the smooth muscle cells are embedded, can influence the phenotypic state of the cells. [8] The matrix

constituents heparansulphate-glycosaminoglycans, [9,10,11] laminin and collagen type-IV [12] prevent both endothelial cells and smooth muscle cells from dividing and migrating and keep SMC in a contractile state, collagen-I promotes the synthetic state. [13] Disruption of the contact between smooth muscle cells and these matrix proteins after injury may initiate the phenotype switch.

-Interaction with growth factors. After vessel wall injury, several growth factors that were stored within the extracellular matrix and intracellularly in the endothelium and smooth muscle are liberated. Basic fibroblast growth factor (bFGF) is the most important one, and contributes to the switch from quiescent to synthetic phenotype. [14,15,16,17]

-Interaction with platelets and leukocytes. When the endothelium is damaged, the sub-endothelium becomes exposed. The exposed extracellular matrix contains proteins such as fibronectin, collagen type-I and tissue factor, that may bind and activate circulating platelets and monocytes. [18] They are important sources of growth factors and cytokines that promote the switch of smooth muscle cells phenotype. [19,7]

In conclusion: activation of the contractile smooth muscle cell resulting in the transformation to the synthetic phenotype is the result of several signals from the injured environment of these cells.

MIGRATION OF SMOOTH MUSCLE CELLS

The migration of vascular smooth muscle cells from the tunica media into the neointima is a key event in the development and progression of many vascular diseases. In *in vivo* experiments, migration is a highly predictable consequence of mechanical injury to the blood vessel. Proliferation and migration sometimes overlap in time, but they are two distinguished, and separately regulated processes, as has been shown both *in vivo* [20,21] and in cultured smooth muscle cells. [22] After induction of thrombocytopenia, or after inhibition of platelet activation, the smooth muscle migration is decreased, but not the smooth muscle cell proliferation after vessel wall injury. Inhibitors of DNA synthesis do not abolish smooth muscle cell migration. [20]

Cell migration is a complex process. We distinguish four separate factors that contribute to the process of smooth muscle cell migration: 1- the interactions between the cell adhesion molecules and neighbouring cells or the extracellular matrix, 2- extracellular proteolysis, creating a passage through the three-dimensional extracellular matrix, 3- motility or random movement of the cell over a substrate, 4- chemotaxis or directed movement towards a chemoattractant or against a chemotactic gradient over the vessel wall.

Ad 1

Adhesive proteins form a connection between cells and their surrounding extracellular matrix. These cell adhesion molecules are involved both in cell-cell interaction and cell-extracellular matrix interaction. Their occurrence on smooth muscle cells is summarised in TABLE 1.

Integrins contain an α and a ß-domain; several variant sub-types (12 α and 8 ß variants) of these domains are presently known; [23] they determine the specificity towards the ligand that is bound by the integrin. Not much is known about the regulation of integrins in relation to migration of smooth muscle cells, but three possible mechanisms are: (1) detachment of the integrin from the ligand; (2) internalisation of integrins; (3) up- or down-regulation of integrin domain synthesis.

Regarding the first mechanism: Disruption of integrin-matrix interaction by G4120, a cyclic RGD-containing peptide results in a reduction of neointima formation after vessel wall injury in hamsters. [24] Thrombospondin is believed to play a role in destabilising the interaction between integrins and the matrix molecules. [23] Contact between ductus arteriosus smooth muscle cells and collagen type-I is mediated by integrins α1 ß1 and α6 ß1, this interaction can be interrupted by laminin. [25]

Regarding the third mechanism: Smooth muscle cell expression of α1 ß1 and α2 ß1 integrin receptors for collagen and laminin is dynamic; their relative contribution changes when smooth muscle cells start to change their phenotype, this regulates SMC migration over a type I collagen substrate. Quiescent smooth muscle cells in the media of normal arteries express α1 ß1 integrin, but no detectable α2 ß1, in proliferating

TABLE 1. Adhesion molecules on vascular smooth muscle cells

Adhesion molecule	Ligands	References
α1 ß1-integrin	laminin, collagen-I and collagen-IV	(196, 197, 198, 199, 200)
α2 ß1-integrin	fibronectin, laminin, collagen-I and collagen-IV	(196, 197, 198, 199, 200)
α3 ß1-integrin	fibronectin	(196, 197, 198, 199, 200)
α5 ß1-integrin	fibronectin, gelatin, denatured collagen	(196, 197, 198, 199, 200)
αv ß1-integrin	fibronectin	(196, 197, 198, 199, 200)
αv ß3-integrin	vitronectin, fibrinogen, vWF, osteopontin, thrombospondin, gelatin, fibronectin (any protein with RGD-sequence)	(197, 200)
VCAM-1	α4ß1-integrin or platelet VLA 4	(199, 196)

Summary of cell adhesion molecules found in vascular smooth muscle cells with their principal ligands. The numbers refer to the literature in the reference section. VCAM=vascular cell adhesion molecule; vWF=von Willebrandt factor

SMC cultured from arterial mediae, no α1 ß1 integrin, but a strong expression of α2 ß1 is found. The latter integrin is required for migration over a collagen-I substrate. [26] Vitronectin increases directed human aortic SMC migration in a Boyden chamber assay; the cell migration is mediated by αV ß3 integrin; a vitronectin receptor. Expression of the receptor and resulting migration are increased by TGF-ß and thrombin, and inhibited by cAMP. [27,28] PDGF is reported to modulate the phenotype of SMCs by altering fibronectin-integrin interaction, possibly by regulation of the fibronectin receptor. [29] These data show that a change in expression of integrins can modulate cell migration.Integrins are also involved in the interaction between smooth muscle cells and platelets or monocytes, that can increase the local levels of many growth factors; in clinical trials antibodies directed against platelet integrins reduced arterial thrombosis and restenosis after percutaneous coronary interventions. [30]

Ad 2

In vivo, smooth muscle cells are surrounded by and embedded in a variety of extracellular matrix (ECM) proteins, that must be traversed during migration. One of the principal barriers to cell movement in the intact vessel is the basement membrane that separates the SMC-containing medial cell layer from the endothelium. It contains collagen type-IV, laminin and heparansulphate proteoglycans. The space between smooth muscle cells is occupied with extracellular matrix proteins, consisting mainly of collagen types I and III, fibronectin, chondroitinsulphate and dermatansulphate proteoglycans and elastin. [31,32,8] Migration involves the excretion and activation by activated smooth muscle cells of extracellular proteases that disrupt the basement membrane and degrade extracellular matrix proteins so as to enable migration through this three dimensional network of proteins. The system of extracellular proteolysis, which is activated during the process of migration, will be discussed in a separate section.

Ad 3

Some cytokines and growth factors are reported to modulate the motility of smooth muscle cells. Several platelet factors increase the migration of bovine aortic SMC over a collagen type-I coated surface: serotonin, PDGF, TGF-ß, norepinephrine and histamine all increase smooth muscle cell migration, while decreasing endothelial cell migration. [33] Ang II increases migration [34,35] of bovine aortic SMC over a collagen type-I coated surface; the effect is abolished by the angiotensin receptor inhibitor Saralasin. PDGF stimulates the migration of bovine smooth muscle cells and its effect is partially mediated by the induction of endogenous bFGF. [36] The motility of smooth muscle cells cultured from atherectomy material is higher from patients who have developed restenosis, than cells derived from patients with a primary stenosis. [37,38] It appears that these intimal smooth muscle cells acquired a permanently increased

motility, which is correlated with an increased risk of a second stenosis after surgery. Plasminogen activation activity, particularly urokinase, is reported in most migrating cell types. [39,40,41] There is accumulating evidence that plasminogen activation is important during smooth muscle cell migration, not only because it allows migration by degradation of the three-dimensional matrix surrounding the cell, but also by enhancing random motility over a flat surface, which is essentially a two-dimensional process. [31,42,43,44,45]

Ad 4

A chemotactic gradient over the vessel wall is created when platelets attach to the damaged intima after injury and start to release PDGF which is a very potent chemotactant for smooth muscle cells. [38,46,47,48,44] Both PDGF-BB (10 ng/ml) and platelet releasate are chemotactic for rat carotic artery medial smooth muscle cells. TGF-ß, another platelet mitogen is hardly chemotactic, [49,50] although it is able to increase chemokinesis or random cell motility. [33] In thrombocytopenic rats, medial SMC replication rates 6 hours after balloon catheter denudation were comparable to control animals, but the migration of SMC from the media to the intima and the extent of intimal thickening were reduced. This indicates that directed migration of medial smooth muscle cells is dependent on platelet products, but proliferation of these cells is not. [47,48]

Basic FGF increases the directed migration of SMC by 50%. [49] Thrombospondin is another potent chemoattractant of SMC. It can potentiate chemoattraction by PDGF. PDGF also induces thrombospondin synthesis by SMC, so thrombospondin may start an "autocrine chemoattraction cascade": smooth muscle cells are attracted by PDGF and start synthesising thrombospondin that will attract other SMC. [51]

EXTRACELLULAR PROTEOLYSIS IN MIGRATING SMOOTH MUSCLE CELLS

Degradation or turn-over of extracellular matrix proteins is an important step in the migration process. The activity of extracellular proteases is increased in migrating smooth muscle cells and this may result both in detachment of the cells' anchoring points from their substrate and in creating a passage through the basement membrane and extracellular matrix. This enables smooth muscle cells to migrate through the vessel wall.

The main two classes of proteinases involved in extracellular proteolysis are the plasminogen activators and the metalloproteinases. Co-ordinate and consecutive action of plasminogen activators, plasmin and metalloproteinases is well documented during

angiogenesis, [52,53] tumour cell invasion and metastasis, [54,55,56] wound healing, [57,58,59] uterine remodelling [60,61] and ovulation, [62,63] and appears to be important in the development of atherosclerosis as well. [64,65,31,44] We will summarise the plasminogen activation system and the metalloproteinase system and will then discuss what is known about these classes of proteases with respect to smooth muscle cell migration.

The plasminogen activation system

The plasminogen activation system, schematically represented in FIGURE 1, involves the following components:

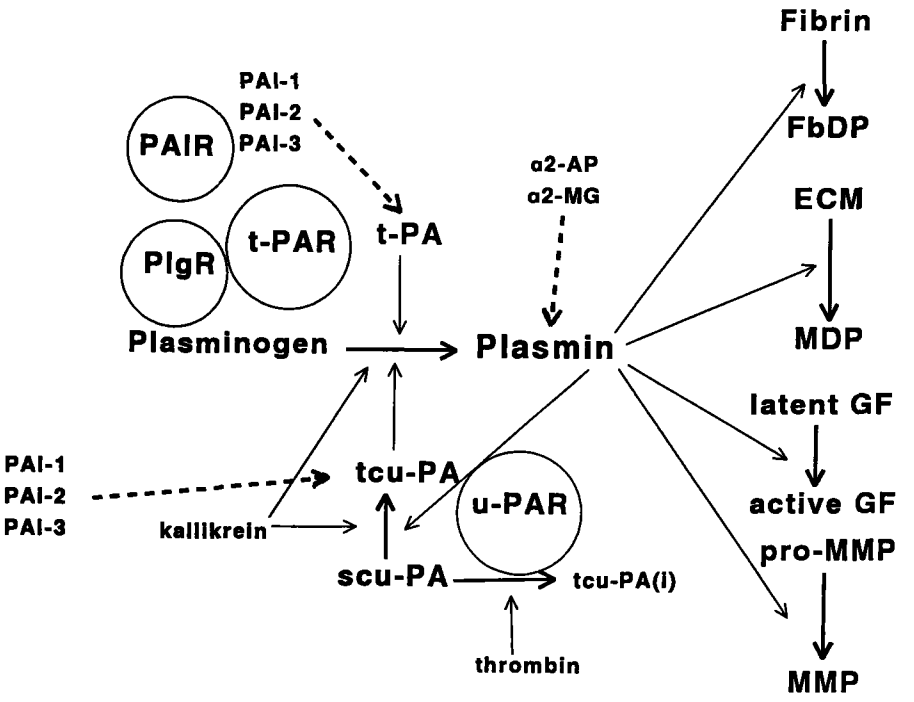
-Plasminogen is an abundantly circulating zymogen that can be activated by both tissue-type and urokinase-type plasminogen activator to yield active plasmin. [66] A main function of plasmin is to degrade fibrin clots. [67,68] Moreover plasmin is able to degrade several matrix constituents: collagen type-IV and type-V, proteoglycans, laminin, fibronectin, [69] but it can also activate latent metalloproteinases and elastase, [70,52,54] as well as latent growth factors such as TGF-ß. [71,72] Finally the mitogen basic FGF which is inactive when matrix-bound, can be released by plasmin activity. [73]

-Plasmin can be inhibited by its principal inhibitor α2-antiplasmin, but also by α2-macroglobulin and α1-protease inhibitor; [74] normally very little active plasmin is present in the circulation. Plasminogen activation is facilitated after the binding of plasminogen to fibrin, thrombospondin, extracellular matrix, or certain cell surface receptors. [75,76] Receptor-bound plasmin is protected against its inhibitors. [77]

-Tissue-type plasminogen activator or t-PA is produced in an active form, it possesses an affinity for fibrin, and its efficacy to convert plasminogen is markedly enhanced in the presence of fibrin. [78] In addition t-PA will bind to some cell surface receptors such as the 40 kD phospholipid binding protein annexin II and enolase. Annexin-II (present on endothelium cells) and enolase (on monocytes) are receptors for both t-PA and Plg, and just like fibrin they give rise to the formation of a ternary complex that enables very efficient activation of plasminogen. [79,76] The t-PA gene expression, recently reviewed by Kooistra *et al,* [80] is increased amongst others by cytokines such as IL-1, TNF-α, by bacterial lipopolysaccharides, retinoids (vitamin A derivatives), and is inhibited by TGF-ß and heparin. [81,82]

-Urokinase-type plasminogen activator or u-PA is excreted in an inactive single-chain form (scu-PA or pro-urokinase) and must be pre-activated by plasmin, or less efficiently by activated kallikrein. Pro-urokinase can be degraded by thrombin. The plasmin mediated activation is much more efficient after pro-urokinase binds to the cellular u-PA receptor. [41] The scu-PA.uPA R complex and the plasmin-receptor complex interact on the cells' surface and ensure an accelerated activation of scu-PA. The u-PA gene has a cAMP-consensus element, it is induced by di-butyryl-cAMP and forskolin. [83] It is further induced by tumour promoters (phorbol esters), growth factors, glucocorticoids, and is inhibited by dexamethasone. [66,84] Urokinase and t-PA both play their role in the

FIGURE 1. Schematic representation of the plasminogen activation system.



PAI: plasminogen activator inhibitor; t-PA: tissue-type plasminogen activator; scu-PA, tcu-PA: single-chain respectively two-chain urokinase-type plasminogen activator; PN: protease nexin; α2-AP: α2-antiplasmin; α2-MG: α2-macroglobulin; t-PAR: cellular t-PA receptors, such as annexin II and enolase; u-PAR: cellular u-PA receptor; PlgR: cellular plasminogen receptor; PAIR: PAI-1 receptor, such as vitronectin; tcu-PA(i): inactivated urokinase; FbDP: fibrin degradation products; ECM: extracellular matrix proteins; MDP: degradation products of extracellular matrix proteins; MMP: active form of matrix metalloproteinases; GF: growth factors e.g. TGF-β; PAI-3 is also known as protein-C inhibitor.

degradation of fibrin and thrombi in vivo, as has been shown with transgenic mice deficient in one or both of the plasminogen activators. Inactivation of the t-PA gene impairs clot lysis and inactivation of the u-PA gene results in occasional fibrin deposition in the mice, while plasminogen deficiency or combined t-PA and u-PA deficiency cause extensive spontaneous fibrin deposition. [85,86] There is still some controversy if other plasminogen activators apart from t-PA and u-PA exist; kallikrein is reported to be an activator of plasminogen, another candidate is the still unidentified plasminogen activator that has been postulated by Binnema *et al.* [87] Their role in fibrin degradation seems to be limited though, as indicated by the similar phenotype of combined t-PA/u-PA (-/-) and plasminogen deficient mice. [85]

-Different inhibitors of plasminogen activators are known, they are summarised in TABLE 2.

--Plasminogen activator inhibitor (PAI)-1 is the most important one that can inactivate both t-PA and u-PA. [88] PAI-1 is synthesised by many cell types: [89] endothelial cells, [90,91] smooth muscle cells, [92,93] hepatocytes, [94] fibroblasts [95] and certain tumour types. [83,96] The synthesis of PAI-1 is induced by stimuli as serum, endotoxin, TNF, interleukin-1, endotoxin, insulin, PDGF, Ang II, thrombin, dexamethazone, serotonin and TGF-ß. [97] The concentration of PAI-1 is 15-30 ng/ml in plasma, but around 5 times more is present in circulating platelets. [98,88] PAI-1 is present in high concentrations in the extracellular matrix, bound by vitronectin and heparansulphates. [99,100,20]

--Plasminogen activator inhibitor-2 is one order of magnitude less efficient than PAI-1 in inhibiting t-PA, it is present in endothelial cells, [101] keratinocytes, [102] monocytes/macrophages, [103,88,104] but also in many tumour cell lines. [96,83,66] Its activity in circulation is only significant during pregnancy. The function of a substantial intracellular pool of PAI-2 found in many cell types is at present unclear. [105]

--Plasminogen activator inhibitor-3 or protein C inhibitor is three orders of magnitude less efficient than PAI-1 as an inhibitor of plasminogen activators, but it is present in high concentrations in the circulation (5 μg/ml). [106]

--Protease nexin is a fast acting inhibitor of thrombin, with some preference for urokinase and tissue-type plasminogen activator and plasmin. Its distribution is mainly limited to the brain vessels. [107]

--C1-inhibitor is an inhibitor of the complement activation cascade, present in high concentrations in circulation, that also weakly inhibits the fibrinolysis system. [108,109]

--Alpha-2 proteinase inhibitor, or α2-antitrypsin is a serpin with a molecular weight of 55 kD. It is a poor inhibitor of both plasmin and t-PA. [105,108]

-- Alpha-2 macroglobulin (α2-MG) is a tetrameric protein (4 x 180kD) that binds many types of active proteases including plasmin, t-PA and u-PA. These three inhibitors (C1-inhibitor, α1-antitrypsin and α2-MG) are all present in very high concentrations in the plasma, but their ability to bind plasminogen activators is at least 4 orders of magnitude lower than PAI-1 (TABLE 2). [110,111]

-Several types of receptors involved in clearing plasminogen activators from the circulation are known.

--Mannose receptors present in liver endothelial cells bind the mannose in the oligosaccharide sidechains in t-PA and u-PA and effectively clear plasminogen activators from circulation. [112,105]

--A fucose-receptor present on cultured hepatoma cells recognises the fucose residue on Thr 61 in t-PA. [113]

--The low-density lipoprotein receptor-related protein or α2-macroglobulin receptor (LRP/α2-MR) is a large glycoprotein (MW 600 kD) present on smooth muscle, but not

TABLE 2 Inhibitors of the plasminogen activation system, their plasmaconcentrations in (M) and the efficiency of binding, expressed as reaction constant of dissociation (k_2 in $M^{-1}.s^{-1}$).

Inhibitor	Concentration (ml^{-1} plasma)	k_2(with tsu-PA)	k_2(with sct-PA)	k_2(with tst-PA)	k_2(with plasmin)
PAI-1	15-30 ng	$1.8 .10^7$	$5.5 .10^6$	$1.8 .10^7$	
PAI-2	0-250 ng	$2.1 .10^6$	$4.6 .10^3$	$1.2 .10^5$	
PAI-3	5 μg	$8 .10^3$	$1 .10^3$	$1 .10^3$	
C1-inh	1.7 μg			<100	
α1-PI	23.5 μg			<100	
α2-AP	1 μg		50	50	$2 .10^7$
PN	0	$2 .10^5$	$1 .10^5$	$3 .10^4$	
α2-MG	3 μg			<100	$5 .10^5$

tcu-PA: two-chain form of urokinase-type plasminogen activator; sct-PA: single chain form of tissue-type plasminogen activator; tct-PA: two-chain form of tissue-type plasminogen activator; PAI: plasminogen activator inhibitor; PAI-3 is also known as protein-C inhibitor; C1-inh: complement-1 inhibitor; α1-PI: α1-proteinase inhibitor or α1-antitrypsin; α2-AP: α2-antiplasmin; PN: protease nexin; α2-MG: α2-macroglobulin.

on endothelium. It is involved in the internalisation of α2-macroglobulin.protease complexes, apoB and apoE particles, [111] pro-urokinase, [114] u-PA.PAI-1 complex, [115,116] and t-PA.PAI-1 complex. [115,112] It is still a matter of controversy whether free t-PA binds to LRP too, [115,117,118] but the evidence is accumulating that it does. [76,119] The α2-macroglobulin receptor-associated protein (RAP) inhibits the interaction between LRP with its ligands [120,121,115] and can inhibit internalisation of plasminogen activator.PAI complexes.

Involvement of plasminogen activation in smooth muscle cell migration

Vascular smooth muscle cells can be induced to produce most components of the plasminogen activation system: tissue-type and urokinase-type plasminogen activator, [43] their inhibitor PAI-1, [122,123,124] the urokinase receptor [125,126] and the receptor LRP/α2-MR, involved in clearance of t-PA, u-PA and PA/PAI complexes. [117]

The local activity of t-PA and u-PA is regulated by rate of synthesis, excretion, binding to receptors, internalisation and degradation as well as inactivation by specific inhibitors. In the rat aortic media a basal t-PA activity is present, but little or no urokinase activity. [127] Both tissue-type and urokinase-type plasminogen activator activity were found increased in the rat aorta after injury. [44,42] Reilly *et al* found differences in expression of t-PA and u-PA between healthy human aortae and dilating or occluding

aortae. [128] In intimal smooth muscle cells cultured from injured baboon aortas, u-PA activity was increased after 4 days. [129] Both urokinase plasminogen activator (u-PA) and t-PA activity are increased in injured rat carotid arteries. The increase reaches a maximum after 7 days. [82]

Proliferating SMC display a high u-PA activity, while migrating cells show mainly t-PA activity. [7] A strong increase in tissue-type plasminogen activator activity is found 4-8 days after injury and this coincides with the main wave of cell migration. At the same time a smaller, but significant increase in u-PA is observed. The increase in t-PA activity is partly mediated by PDGF, since inhibiting antibodies against PDGF attenuated the increase in t-PA activity in injured blood vessels. [44] Thrombin is another substance that causes an increase in tissue-type plasminogen activator (t-PA) levels in smooth muscle cells of different origin. [130]

Plasminogen activation is involved in the migration of smooth muscle cells after injury of the vessel wall. Tranexamic acid (an inhibitor of plasminogen activation) reduces the rate of smooth muscle cell migration in the rat *in vivo* [42] and the intimal area invaded by migrating smooth muscle cells, indicating that plasmin generation is necessary for migration. Attenuation of the increase in plasminogen activator activity may inhibit the migration of smooth muscle cells towards the intima. Heparin represses the expression of t-PA, [131,81,82,81] but not of u-PA, after injury in the vessel wall or after stimulation of smooth muscle cell cultures with phorbolester. Heparansulphates and heparin can inhibit the SMC proliferation and migration. The reduction in t-PA activity by heparin may be the cause of this reduced migration. However, the inhibitory effect of heparin are caused by decreasing activator protein-1 (AP-1) binding to DNA, this affects all processes that are c-fos/jun mediated, making it difficult to demonstrate that it is the decrease in t-PA activity that causes the reduction in SMC migration. [132]

The urokinase-type plasminogen activator receptor (u-PAR) was demonstrated on cultured smooth muscle cells of bovine aorta. Alpha-thrombin and other mitogens (EGF, TGF-ß1, basic FGF, PDGF) as well as phorbol esters, increase u-PAR expression on smooth muscle cells. [126,133] The increase in urokinase activity and number of u-PA receptors in proliferating smooth muscle cells potentially reinforces the role of t-PA in matrix degradation and migration of smooth muscle cells in the injured vessel wall.

Local production of plasminogen activator inhibitors by smooth muscle cells but also by endothelium and mononuclear cells can modulate the local plasminogen activator activity. PAI-1 mRNA and protein were increased in endothelial cells, in smooth muscle cells and in macrophages juxtaposed to thrombi induced in rabbit carotid arteries. [134] PAI-1 mRNA was increased in endothelial and smooth muscle cells adjacent to the neointima of injured rat aorta. [134] SMC in culture respond to thrombin treatment with an increase in plasminogen activator inhibitor type-1 (PAI-1) levels. PAI-2 levels in cell lysates of such SMC are not affected by thrombin. [130] Growth factors such as

PDGF, EGF and TGF-ß1 increase PAI-1 protein in conditioned medium and in ECM of cultured bovine SMC. [92,135] From these data it emerges that many of the factors found in or near the injured vessel wall increase plasminogen activator inhibitor activity. It must be noted that the same growth factors (e.g. PDGF and thrombin) increase the production of both plasminogen activator and PA-inhibitor, and in addition, thrombin is able to inactivate urokinase. The net effect on plasmin activity may depend on local circumstances. The importance of the simultaneous production of both plasminogen activators and their inhibitors may be to restrict proteolysis to a very limited area. There is a focal plasminogen activator activity, combined with a surplus of inhibitor in the immediate vicinity, in order to limit over-activity of proteolysis.

Experiments with transgenic mice support the involvement of the plasminogen activation system in cell migration through the vessel wall. [136] This has been shown both in 1- the invasion of metastatic tumour cells and in 2- the neointima formation in the injured mouse arteries.

-1- Metastasis requires the migration of tumour cells through the vessel wall, plasminogen activation is involved in this process, as has been shown using transgenic mice. The number of Lewis lung carcinoma metastases in transgenic mice expressing high levels of human PAI-1 was markedly reduced, but not the growth of the primary tumour. [137] This suggests that inhibition of plasminogen activator activity inhibits tumour cell migration through the vessel wall.

-2- Involvement of plasminogen activators in the migration of medial smooth muscle cells to the neointima after injury of the carotic artery in a transgenic mouse model was shown by several groups. The *extent* of neointima formation in mice carotid artery after injury was not significantly altered in t-PA deficient (-/-), or PAI-1 (-/-) transgenic mice, but it was significantly reduced in u-PA (-/-), and in combined u-PA/t-PA (-/-) mice. [138,136] The *rate* of neointima formation was increased in PAI-1 deficient transgenic mice and reduced in u-PA (-/-), while neointima formation in t-PA deficient mice was comparable to wild type. [139,85] This suggests an important role for plasminogen activators, especially urokinase in the migration of cells through the vessel wall after intimal injury, which coincides with data from Jackson *et al,* [44] who reported that inhibitors of plasminogen activation could effectively inhibit SMC migration through the injured rat carotid artery.

Summarising: plasminogen activators are involved in the process of medial SMC migration through the vessel wall. From experiments with plasminogen activator deficient transgenic mice, it seems that urokinase is the most important component.

The matrix metalloproteases

Matrix metalloproteinases (MMP's) are a class of genetically related endoproteinases characterised by the presence of an Zn-ion in the active centre and the need for an additional Ca-ion. [140] Over ten members of this family of proteases are known, often under several synonyms (TABLE 3). MMP's are involved in the degradation of many extracellular matrix proteins [53,141] (TABLE 4).

The activity of matrix metalloproteinases can be modulated by the rate of synthesis (gene-regulation), by activation of the proenzyme and by interaction with inhibitors.

The regulation of MMP synthesis by smooth muscle cells is discussed in a separate section. Metalloproteinases are secreted as a pro-enzyme or zymogen and can be activated by cleaving off a 10 kD propeptide. Activation of pro-MMPs can be caused by various proteases including plasmin, [52,70] stromelysin or MMP-3, [53,54] trypsin, kallikrein, chymotrypsin. [54,142,143] MT-MMP is a newly reported membrane-bound metalloproteinase that is potent in activating MMP-2. [144] Another metalloproteinase activating pro-MMP-9 and pro-MMP-8 has recently been described. It does not activate pro-MMP-2 and can be inhibited by TIMP-2. [145] Active oxygen (HOCl, H_2O_2, HO• present in inflammation) can activate pro-MMP-8. Autocatalytic activation of pro-MMP-2 and pro-MMP-9 is known to occur in an acid environment, e.g. in gastric juices. [146,147]

Several inhibitors of metalloproteinases have been reported. MMP's are inhibited by specific tissue inhibitors of metalloproteinases: TIMP-1 and -2. [148,149] Another inhibitor: α2-macroglobulin (α2-MG) is a slow-acting, non-specific, irreversible inhibitor of active proteases of all classes, including metalloproteinases. The interaction with TIMP-1 is very fast and gives a tight equimolar complex with all MMPs [141,150,151] TIMP-1 is heavily glycosylated (30% of its molecular weight), it does not bind to pro-MMPs, but only to active MMPs (there is some controversy over this). TIMP-1 itself is inactivated through cleavage by MMP-1, trypsin, and α-chymotrypsin. TIMP-1 seems to be identical to the previously described haemocytokine "erythroid potentiating activity", but also TIMP-2 has erythroid potentiating activity. [152,153,148,154] Plasmin is insensitive to TIMP-1 or TIMP-2. [155] TIMP-1 binds to heparin/heparansulphates; [151] it is found in extracellular matrix associated fractions from human aorta. [156] TIMP-2 is a very similar but non-glycosylated inhibitor of MMPs, with a preference for MMP-2. [149,157,158]

Involvement of metalloproteinases in smooth muscle cell migration

Recently it became known that vascular smooth muscle cells produce several proteases of the MMP family. The knowledge of this MMP system and its involvement in smooth muscle cell migration is rapidly expanding. We will discuss here the production of MMP-1, -2, -3, and -9, and the inhibitors TIMP-1 and -2 by smooth muscle cells and their role in smooth muscle cell migration.

TABLE 3. The matrix metalloproteinases (MMP), properties and synonyms.

Type	Mol weight active enzyme[1]	Synonyms, activation and cellular sources[2]
MMP-1	(57) 48	Type-I collagenase, neutrophil or fibroblast collagenase. Activated by plasmin, trypsin, chymotrypsin, stromelysin. Induced by AP-1 element (159). Produced by fibroblasts but also chondrocytes, PMN, monocytes and macrophages, endothelial cells, tumour cells, keratinocytes and SMC.
MMP-2	(72) 66	Type-IV collagenase, 72 kD-gelatinase, gelatinase-A. Activated by membrane-bound MT-MMP (144), not by plasmin or trypsin. Constitutively produced by e.g. endothelial cells, chondrocytes, PMN, tumour cells, monocytes and macrophages, fibroblasts, SMC.
MMP-3	(57) 48	Stromelysin, transin, proteoglycanase. Activated by elastase, cathepsin G (201), plasmin (151,125). Produced by e.g. endothelial cells, chondrocytes, tumour cells, keratinocytes, macrophages, fibroblasts, SMC.
MMP-4		Telopeptidase from human gingival tissue
MMP-6		From human cartilage
MMP-7	28	PUMP-1. In rat uterus cells.
MMP-8	(75) 67	PMN collagenase, neutrophil collagenase. Activated by trypsin, chymotrypsin. Stored intracellularly in polymorphonucleate cells.
MMP-9	(92) 88	Type-IV collagenase, 92 kD gelatinase, gelatinase-B, monocyte or saliva gelatinase. Activated by elastase, cathepsin G, plasmin (140), auto cleavage, trypsin, stromelysin (142), not by MT-MMP. In e.g. endothelial cells, chondrocytes, PMN, tumour cells, monocytes and macrophages, fibroblasts, SMC.
MMP-10	53	Stromelysin-2, transin-2. In tumour cells
MMP-13		Collagenase-3, murine collagenase
MT-MMP		Membrane-type MMP, activator of other MMPs

[1]The molecular weight of the pro-enzyme in parentheses. [2]The numbers in parentheses refer to the literature in the reference section. PMN=polymorphonuclear leukocytes; SMC=smooth muscle cells; PUMP=putative metalloprotease

-MMP-1. In serum free cultures of smooth muscle cells isolated from the human aorta, (virtually) no basal collagenase activity was detected, but in the presence of PDGF, IL-1 or TNF- [159,160,161] collagenase (MMP-1) activity was detectable. Similarly in cultured baboon aortic SMC, no MMP-1 expression was found in serum-free culture conditions, but after the addition of serum or phorbol ester, MMP-1 mRNA was strongly increased. [162] Kishi [163] reported the production of interstitial collagenase (MMP-1) in bovine aortic SMC. In smooth muscle cells from rabbit aorta, grown in serum free medium, no collagenase (MMP-1) activity or antigen was detected, but no data were available after serum induction. [65] In cultures of human aortic medial smooth muscle cells, small

amounts of proMMP-1 and -3 were detected. In intimal smooth muscle cell cultures the amounts of these proenzymes exceeded those found in the medial smooth muscle cells. [164]

TABLE 4. Enzymatic degradation of extracellular matrix proteins with the principal responsible proteases.

Collagen I or interstitial type collagen	MMP-1, MMP-3, MMP-8, MMP-13
Collagen II	MMP-1, MMP-3, MMP-8
Collagen III	MMP-1, MMP-3, MMP-8, MMP-10
Collagen IV	MMP-2, MMP-3, MMP-9, MMP-10, elastase, trypsin, plasmin
Collagen V	MMP-2, MMP-3, MMP-9, MMP-10, (trypsin, plasmin, MMP-8 inefficiently)
Collagen VII	MMP-1, MMP-2
Collagen IX	MMP-3
Collagen X	MMP-1, MMP-3
Collagen XI	MMP-2
Gelatin (denatured collagens)	MMP-2, MMP-9, MMP-10, MMP-7, (MMP-1, MMP-3 and plasmin inefficiently)
Elastin	MMP-2, MMP-3 (MMP-9), neutrophil elastase, (cathepsin-L inefficiently)
Glycoproteins	Numerous proteases, specificity differs according to molecule
Proteoglycans	Glycanases (the carbohydrate part), in positive co-operation with MMP-3, MMP-7, plasmin, elastase (the protein core)
Laminin	MMP-3, plasmin
Fibronectin	MMP-2, MMP-3, MMP-7, MMP-10, plasmin, t-PA

The above extracellular matrix constituents can be degraded by the indicated extracellular proteolytic enzymes. MMP=matrix metalloproteinase; t-PA= tissue-type plasminogen activator.

The regulation of the interstitial collagenase (MMP-1), is well characterised. The promoter of the MMP-1 gene in skin fibroblasts contains a phorbol ester (TPA)-responsive element (TRE), that is directly stimulated by phorbol-esters and by the dimeric transcription factor c-jun/c-fos (or AP-1). [165] MMP-1 expression is increased by phorbol esters in cultured human aortic smooth muscle cells. [160] Some cytokines that increase protein kinase-C activity are known to increase collagenase expression in smooth muscle cells: PDGF, TNF-α and IL-1 [160,161] increase MMP-1 mRNA in cultured human aortic medial smooth muscle cells. Recently it was found that the murine

collagenase is not homologous with the human MMP-1, [166] but with the human collagenase MMP-13, it does, however display the same TRE element as MMP-1.
The genes for MMP-3, [167] MMP-9 [168] and TIMP-1 [169] have TRE-like sequences in their promoter regions, and are expected to be regulated by the same protein kinase-C mediated factors as MMP-1. The MMP-1 promoter also contains a transacting TGF-inhibitory element (TIE): TGF-ß inhibits MMP-1 transcription via this TIE-element upstream from the TRE element. [53,64,170] This TGF-inhibitory element is present on the MMP-3 and MMP-9 promoters as well, but it is absent from the MMP-2 promoter. [64]

MMP-2. Zempo *et al* [171] reported the constitutive expression of a 72 kD type IV collagenase (MMP 2) in normal carotid arteries. The activated form of this enzyme was increased 5 days after catheterisation, when SMC started to migrate.
MMP-2 was found in the conditioned medium of human arterial smooth muscle cells, [172,173] human aortic smooth muscle cells, [159] intimal smooth muscle cells cultured from injured baboon aortas [129] and smooth muscle cells from rabbit aorta. [65]
MMP-2 does not appear to have TRE-sequences in its promoter-enhancer regions [174] and behaves different from MMP-1. Matrix metalloproteinase 2 was found to be constitutively expressed in cultured human aortic SMC and its synthesis was not affected by PDGF, interleukin 1 or phorbol esters. [160]

MMP-3. Small amounts of proMMP-3 are present in cultures of medial smooth muscle cells. Cultured intimal smooth muscle cells produce higher concentrations of this proenzyme. [164] Synthesis of MMP-3 is increased in smooth muscle cells stimulated with interleukin-1 or tumour necrosis factor-alpha. [161] Henney [175] demonstrated the expression of stromelysin (MMP-3) mRNA in human atherosclerotic plaque. Some expression was seen in smooth muscle cells, but more extensive expression was associated with macrophages. Recently we found mRNA for MMP-3 in cultured rat aortic SMC that had been stimulated with PDGF-BB. [176] In another report, metalloproteinase 3 (stromelysin) was not detected in cultured human aortic medial smooth muscle cells, in basal conditions, or after treatment with PDGF, interleukin-1, prostaglandin-E2 or the phorbol ester TPA. [160] This is in contrast to the induction of the MMP-3 mRNA after PDGF treatment of cultured RASM, [176] and after IL-1 treatment of human vascular smooth muscle cells. [161] The discrepancy can not be explained, but could be due to low detection limit of MMP-3 antigen. [160] TGF-ß decreases MMP-3 expression. [53]

MMP-9. In smooth muscle cells from rabbit aorta, MMP-9 (or 92 kD gelatinase) was found. [65] MMP-9 was increased 24 hours after injury in rat carotic arteries, and decreased after a few days. [171,31] In intimal smooth muscle cells cultured from injured baboon aortas, MMP-9 activity was detectable 3-6 days after injury. [129] MMP-9

production in macrophages is increased by phorbol esters and IL-1. [177] Human vascular SMC stimulated with interleukin-1 or TNF-α synthesised de novo MMP-9, and MMP-3, but not TIMP. [161]

TIMP. Tissue inhibitor of metalloproteinases (TIMP)-1 is found in aortic tissue, and is associated with the extracellular matrix. [156] TIMP-1 is constitutively produced by cultured primate SMC. [64,161] TGF-ß increases TIMP-1 gene expression, whereas it decreases MMP-3 and MMP-1 expression. [53]
Also the other inhibitor of metalloproteinases, TIMP-2 is found in aortic smooth muscle cells. [161]

Association of MMP activity and smooth muscle cell migration, atherosclerosis or vessel wall hyperplasia has been established in a number of cases.
Intimal smooth muscle cells taken in culture 1, 2, 3 to 7 days after de-endothelialisation in baboon aorta, show an increase in MMP-2 and pro-MMP-2 activity reaching a maximum between day 5-7. MMP-9 activity was visible from day 6. [129]
MMP-2 activity expressed by serum-starved or contractile smooth muscle cells was less than 5% of that measured in proliferating SMC. SMC migration after vessel injury *in vivo* may be dependent on an increase in MMP-2 activity after the transition from a quiescent and differentiated state to that of a dedifferentiated, proliferating, and motile phenotype. [173]
Bendeck 1994 [31] found an increase in an 88 kD gelatinase activity and the corresponding MMP-9 mRNA in injured rat carotic arteries. A 92 kD gelatinase or MMP-9 was increased 24 hours after injury in rat carotic arteries, when SMC entered the growth cycle, and decreased thereafter. [171]
Extensive expression of MMP-3 mRNA and protein was found in atherosclerotic lesions, moreover, genetic variation in the human MMP-3 promoter region is associated with progression of coronary atherosclerosis. [178,179] A metalloproteinase with elastolytic activity of low-molecular-weight was present in the adventitia, and the activity was increased at 5 days after surgery. [171]

INTERVENTION IN SMC MIGRATION

Intervention in the process of migration of smooth muscle cells after surgery or during the development of atherosclerosis may prevent the progression of vessel wall damage. There are many possible approaches for intervening in SMC migration. Some options have already been explored in *in vitro,* animal or clinical studies, others are mentioned here for their potential efficacy.

-Some inhibitors of SMC random motility have been reported. One of them is N-(3,4-dimethoxycinnamoyl)anthranilic acid or tranilast. The motility of cultured rat vascular smooth muscle cells can be inhibited 50% by tranilast. The mechanism has not yet been elucidated and clinical trials have not yet been reported. [180]

-Angiotensin is known to increase both SMC proliferation and random motility in cell culture, and angiotensin-converting enzyme (ACE) inhibitors inhibit neointima formation in a rat model. Therefore clinical trials with ACE inhibitors have been conducted, but so far with disappointing results. [181,182,183] There seem to be species dependent effects of ACE-inhibitors, as they will reduce neointima formation in the rat, but not in pigs, rabbits, or humans. Increasing the dose of ACE-inhibitor would severely interfere with the blood pressure, so the design of an angiotensin-antagonist that interferes with the SMC migration and proliferation, but not with vasotension could have interesting perspectives.

-Nitric oxide (or EDRF) inhibits smooth muscle cell proliferation and migration, particularly that induced by angiotensin II. [4] Inducers of endogenous EDRF exist (sodium nitroprusside or glyceryl trinitrate), [184,185] but their action is very short-lived and they have strong systemic effects, so they have never been used for the purpose of inhibiting SMC migration after angioplasty.

-Inhibitors of MMP expression include heparin, cyclic AMP analogues and prostaglandins.

Heparin is known to inhibit the interaction between the transcription factor c-fos/c-jun and the AP-1 element, and it is able to inhibit the serum- or phorbol ester-induced increase of MMP-1, -3 and -9 and of t-PA. [131,64,162,81,132] Heparin has been used in clinical trials, but it does not affect restenosis dramatically, [182] although newer heparin preparations with a different molecular weight or degree of sulfatation may have different effects. Preparations of low molecular weight heparin reduced restenosis in animal studies, but this could not be demonstrated yet in clinical trials. [182]

Prostaglandin E2 (PGE2), and iloprost, an analogue of prostacyclin, inhibit the PDGF-induced production of proMMP-1 in cultured human smooth muscle cells in a dose-dependent manner. This suggests that prostacyclins may protect *in vivo* against smooth muscle cell migration and atherosclerosis. [186] In animal studies, a prostaglandin-E1 derivative inhibits intimal thickening in rat arteries after balloon catheter injury. This compound appears to inhibit smooth muscle cell migration through cyclic AMP

elevation. [187] Little clinical data concerning the use of prostaglandins exist. In one trial PGE1 was found to reduce early restenosis after PTCA, but no long term data are available. [182] Agents that increase internal cAMP levels (forskolin, choleratoxin) and cAMP analogues (8-bromo-cAMP, dibutyryl-cAMP), can inhibit growth-factor induced MMP-3 expression. [170] Their use *in vivo* is probably limited, due to the broad spectrum of effects they cause.

-Inhibitors of matrix metalloproteinase (MMP) activity, and antisera neutralising MMP-2 activity inhibit the *in vitro* migration of SMC across an extracellular matrix in a Boyden chamber by > 80%, whereas migration that occurred in the absence of the barrier was unaffected. [173] The MMP inhibitor GM 6001 inhibited SMC migration into the intima almost completely in the rat carotic artery after balloon denudation. [31] The time before proliferating SMC from rabbit aorta explants *in vitro* appeared in the culture dish could be delayed by two structurally unrelated inhibitors of matrix-degrading metalloproteinases, Ro 31-4724 and Ro 31-7467. [65] These data show that SMC migration can be inhibited by inhibitors of MMPs.

- The effect of inhibitors of plasminogen activation such as tranexamic acid, ϵ-amino-caproic acid (t-PA and plasmin), amyloride (u-PA) and inhibitors of metalloproteinases such as the oligopeptide inhibitors, [65,31,188] Galardin MPI, [189] peptidyl hydroxamic acids [190] and phosphoramidon [191] have not been evaluated yet in randomised clinical trials with angioplasty patients. On the basis of *in vitro* data with these inhibitors, these substances have potential efficacy in inhibiting restenosis after angioplasty.

-Local delivery. Many of the aforementioned drugs have adverse systemic effects in addition to the desired effects on the site of vessel injury. Local delivery of these drugs would therefore have many advantages. Two possible methods that enable local delivery of drugs in the arterial wall are an inflatable porous balloon catheter filled with the desired drug [192] and a biodegradable expandable stent impregnated with a pharmacological substance. [193]

CONCLUSIONS AND FINAL REMARKS.

Intimal hypertrophy is a feature of many vasculopathies. The main proliferating cell type is not so much the endothelial, but the smooth muscle cell. Normally the smooth muscle cell does not occur in the intima and migration of the smooth muscle cell from the media to the intima is a key factor in the development of intimal hyperplasia. In order to reach the intima, the smooth muscle cell has to penetrate the basal lamina, the extracellular matrix within the vessel wall, and the lamina elastica interna. It is not dependent on proteolytic enzymes from the circulation or invading leukocytes to digest this matrix, but the migrating smooth muscle cell has the potential to synthesise most of the extracellular proteases required to degrade extracellular matrix proteins. The

production, activation and inhibition of proteases from the plasminogen activation and the matrix metalloproteinase systems are discussed in this review. One important component which is not synthesised by the smooth muscle cell is plasminogen, but this is circulating in very high concentrations in the plasma and is always available. It can be bound to the surface of smooth muscle cells by cell receptors, and the synthesis of these receptors is another important factor in the local fibrinolytic balance. Other proteinases that have not been discussed in this review are the cathepsins and lysosomal proteases. They are largely intracellular enzymes, and their role lies more in the final digestion of fragments of extracellular matrix proteins that have already been pre-digested by plasmin or MMPs. Therefore they are not expected to be a rate-limiting step in matrix degradation and SMC migration. Moreover, cathepsins are active in an acid environment, which limits their role in extracellular matrix degradation as well. [194]

Another enzyme that has not been discussed is elastase. Although elastin is an important component of the vessel wall, not much is known about the production of elastase by smooth muscle cells. SMC do have elastinolytic properties, but this is probably mainly due to the MMP-2 and -3. Sasaguri *et al* [195] demonstrated increased elastinolytic activity in foetal and adult intimal aortic smooth muscle cells compared to adult medial cells, by using a chromogenic substrate. They failed to demonstrate whether this elastinolytic activity is due to elastase, or if it may be caused by metalloproteinases. Monocytic and granulocytic leukocytes are potent producers of elastase, and infiltration of these cell types in the damaged vessel wall, may positively co-operate with the degradation of extracellular matrix by smooth muscle cells.

Many studies have demonstrated the involvement of extracellular proteolysis in the migration of smooth muscle cells, mainly in cell culture and in animal experiments. From these studies a number of possible treating modalities emerged that may prevent primary or secondary stenosis. The extrapolation of these *in vitro* or animal studies to the human situation is not always valid because of the different nature of the strictly controlled model system and the often complicated clinical situation. In addition the relative importance of certain pathways in the animal and in human subjects may be very different, such as the lipid metabolism and the inflammatory cascade; animal studies rarely involve the stenosis in coronary arteries. Data on the clinical effects of protease inhibitors are very limited. Although a plethora of pharmacological agents has been used in clinical trials to assess whether they can prevent restenosis after angioplasty, inhibitors of extracellular proteases have scarcely been evaluated in a clinical setting. There is accumulating evidence however, that extracellular proteolysis plays an important role in SMC migration, which is a key step in the development of atherosclerosis and restenosis.

The administration of inhibitors of plasminogen activation or matrix metalloproteinases shortly after vessel surgery or PTCA, is an interesting new approach to preventing restenosis of the treated blood vessel, and to inhibiting the progression of

atherosclerosis. Hopefully clinical data evaluating the efficacy of these inhibitors will become available in the near future.

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CHAPTER 3.

EFFECTS OF LIPOPOLYSACCHARIDE ON PLASMINOGEN ACTIVATOR INHIBITOR-1 PRODUCTION IN RAT AORTA MEDIA IN VIVO AND IN CULTURED RAT AORTIC SMOOTH MUSCLE CELLS.

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ABSTRACT

Background. Lipopolysaccharide or endotoxin is known to increase the expression of plasminogen activator inhibitor type-1 (PAI-1) in the vessel wall. It is known that endotoxin increases PAI production in endothelial cells, but the role of smooth muscle cells (SMC) is presently not clear. In this study we determined the effect of endotoxin on PAI production by aortic SMC both *in vivo* and in culture.

Methods. The aortae of both Sprague Dawley rats and New Zealand White rabbits were rapidly excised after parenteral administration of endotoxin. Total RNA was extracted from the aortic media and PAI-1 mRNA levels were quantified after Northern blotting. In addition cultured rat aortic SMC were treated with endotoxin. PAI-1 excretion into the conditioned medium was determined with a spectrophotometric assay and total RNA was extracted from the cells and analysed as described above.

Results. A rapid and strong induction in the aortic media of PAI-1 mRNA after parenteral administration of endotoxin in both rat (50 μg/kg intraperitoneal) and rabbit (1 μg/kg intravenous) was observed. Messenger RNA of tissue-type plasminogen activator (t-PA) was barely detectable and could not be increased by endotoxin. Studies in cultured rat aortic smooth muscle cells showed low expression of PAI-1 mRNA under serum free conditions and little PAI activity in the cell conditioned medium. Endotoxin could not increase levels of PAI-1 mRNA or PAI activity under serum free conditions. The effect of addition of endotoxin (10 μg/ml) in the presence of 10% (v/v) newborn calf serum on PAI-1 mRNA was negligible, but PAI activity however, was increased by 50 $\pm$ 7 % compared to controls. PAI activity was identified as PAI-1 by immunoblotting. Fibrin zymography showed that t-PA was present only in complex with PAI-1. Addition of endotoxin (10 μg/ml) in the presence of serum to the cells increased mRNA levels of t-PA and LDL-receptor-related protein/α_2-macroglobulin receptor.

Conclusions. Vascular smooth muscle cells are potent producers of tissue plasminogen activator and plasminogen activator inhibitor-1. The production can be modulated by endotoxin, both *in vivo* and *in vitro*, but the mechanism may be different.

INTRODUCTION

The conversion of plasminogen to plasmin is the key step in the digestion of fibrin and its role in the process of clot lysis is well established. Plasmin however is a serine protease with a broad specificity which is also efficient in cleaving several abundant constituents of the extracellular matrix, as well as in activating several proenzymes of

metalloproteinases. For these reasons a role for plasminogen activation has been advocated not only in fibrinolysis but also in matrix degradation in tissue remodelling, cell migration, and tumor metastasis. [1,2]

The generation of plasmin from its zymogen plasminogen is catalysed by specific enzymes, mainly tissue-type plasminogen activator (t-PA) [3] and urokinase-type plasminogen activator (u-PA). [4] They can be inhibited by their specific inhibitors plasminogen activator inhibitor type-1 (PAI-1), [5,6] and PAI type-2. [7,8] In most cell types *in vivo* basal PAI-1 gene expression is low, but can be increased in stress situations, similar to acute phase proteins [9]. The activity of t-PA is not only regulated by its rate of synthesis and its inactivation by PAI-1, but is also likely to be affected by the rate of internalisation and subsequent degradation in the surrounding cells, or by hepatic clearance. The low-density lipoprotein receptor-related-protein/α_2-macroglobulin receptor (LRP) was recently reported to be involved in internalising PA/PAI-1 complexes, [10,11] as well as free t-PA. [12,13] LRP was found to be present in several cell types, including liver parenchymal cells [13] and vascular smooth muscle cells. [12]

The regulation of plasminogen activation has been studied extensively in cultured human umbilical endothelial cells. They secrete large amounts of t-PA and PAI-1 [14,15] and moreover the induction of PAI-1 mRNA can be increased by several substances, including endotoxin. [16-18] However, little is known about the role of the endothelium *in vivo* and even less is known about the other major component of the vessel wall, the smooth muscle. Simpson *et al.* [19] have shown the presence of PAI-1 antigen in vascular smooth muscle cells *in situ* by immunological staining.

Endotoxin causes a marked increase in PAI activity [20] and PAI-1 mRNA [21] in the rat aortic media, which consists mainly of smooth muscle tissue. Presence of PAI-1 mRNA in the rat aorta media is a very strong argument that PAI-1 is produced by smooth muscle cells. In this study we tried to establish the same induction of PAI-1 mRNA by endotoxin in an other species, namely the rabbit to rule out species specific effects. Endotoxin may directly affect smooth muscle cells in the rat aorta media, but its effect can also be mediated though the endothelium or circulating cells, such as monocytes or macrophages. We performed a series of experiments with cultured rat smooth muscle cells to examine whether the strong induction of PAI-1 mRNA in the aortic media by endotoxin can be explained by a direct effect on aortic smooth muscle cells. In order to get a full picture of the influence of endotoxin on the fibrinolytic potential of cultured smooth muscle we monitored not only the plasminogen activator t-PA and its main inhibitor PAI-1, but also the production of mRNA for urokinase type plasminogen activator (u-PA), PAI-2, plasminogen and LRP, the receptor involved in clearing t-PA from the circulation or the pericellular environment. The fibrinolytic/proteolytic potential of smooth muscle cells (SMC) has important implications for the migration of cells during vessel wall remodelling such as neointima formation, tumor cell metastasis and for the fate of intramural thrombi.

METHODS

Cell culture: Sprague Dawley rat aortic smooth muscle cells were obtained by enzymatic dissociation following the procedure of Gunther *et al.* [22] The cells had a homogeneous appearance, displayed the characteristic hills and valleys appearance and maintained stable characteristics between passages 8 and 15 which were used for the study. SMC were routinely grown in Dulbecco's Modified Eagle's medium (DMEM) (Gibco-Life Technologies, Gaitersburg MD, USA), supplemented with penicillin (100 U/ml), streptomycin (100 mg/L), glutamine (2 mmol/L) and 10% (v/v) new-born calf serum (NCS, Gibco-Life Technologies). Cells used in the experiments were seeded at a density of approximately 10^4 cells/cm^2. Cells were grown to confluence (4-6 days), rinsed twice with serum free medium and kept serum free for another 48 h. The cells were then rinsed twice with serum free medium and medium with the specified agonists was added. The conditioned medium was sampled at specified time intervals and aliquots snap frozen in liquid nitrogen. The cells were used for protein determinations, according to Lowry [23] after dissolution in 0.4 M NaOH, or alternatively, used for RNA extraction after dissolution in guanidinium isothiocyanate (see below).

Animal experiments. Animal experiments were conducted in accordance with Home Office Licences. Sprague Dawley rats weighing 260-300 g were injected intraperitoneally with 50 μg/kg lipopolysaccharide (endotoxin) extracted from E. coli 026:B26 (Sigma Chemical Co., St Louis MO, USA) and killed by cervical dislocation at specified time points. New Zealand White rabbits weighing 2.5-3.5 kg, were given 1 μg/kg of endotoxin by injection into an ear vein. The animals were killed after the specified times with an intravenous injection of sodium pentobarbitone (Euthatal, RMB Animal Health Ltd., Dagenham, U.K.). In both species, control animals received the same volume of vehicle (0.9% saline). Aortae were rapidly excised, the vessels opened longitudinally, the adventitial layer completely removed and the endothelium rubbed off with the blunt end of a scalpel handle. The aortic media were then quickly snap frozen in liquid nitrogen and stored at -70° C. Before RNA extraction, the tissues were pulverised under liquid nitrogen in a mortar, and homogenised in a glass tissue homogeniser.

PAI activity assay. PAI activity in cell-conditioned medium was determined as previously described [24]. Samples were titrated against known amounts of human two-chain melanoma t-PA in the presence of CNBr digested fibrin fragments and plasminogen (Biofine Chemicals, Leiden, the Netherlands). Plasmin generation was assessed by monitoring the cleavage of the chromogenic substrate D-Val-Leu-Lys-p-nitroanilide in a spectrophotometer at a wavelength of 405 nm. PAI activity is expressed in equivalents of international units (IU) of t-PA per mg of cell protein. The

amount of PAI activity detected in non-conditioned 10% NCS medium with or without endotoxin was negligible (data not shown).

RNA extraction, Northern Transfer and Hybridisation. RNA was extracted with guanidinium-isothiocyanate following the method of Chomczynski and Sacchi, [25] quantified at 260 nm in a UV spectrophotometer and its integrity confirmed on an agarose minigel stained with ethidium bromide. Total RNA (5 μg/sample) was electrophoresed on a 1 % agarose gel with 8% formaldehyde and after washing transferred onto a nylon membrane (Hybond N, Amersham International Co., Amersham, Bucks, U.K.). RNA was crosslinked to the membrane using UV irradiation. The PAI-1 probe was a 1.1 kB Pst I fragment of a human PAI-1 cDNA, [26] selected for the highest homology with the rat PAI-1 gene. [27] The t-PA probe was a 2.0 kB Bgl II fragment of the human t-PA cDNA. [15] A 1.2 kB Pst I fragment of the rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal standard. [28] The LRP probe was a 6.0 kB XhoI-EcoRI fragment of human LRP cDNA, [10] kindly provided by Dr. J.Herz. The probes were radioactively labelled with ^{32}P-dCTP using a random primer labelling kit (Megaprime, Amersham, U.K.). Autoradiography was performed on Kodak X-AR film with intensifying screens. The films were digitally scanned and the scanned values for mRNA intensities were normalised for the internal standard glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

Western Blots. 150 μl aliquots of SMC-conditioned medium were subjected to SDS-PAGE and transferred to 0.45 μm nitro-cellulose membrane (Schleicher & Schuell, Dassel, Germany). To saturate non-specific protein binding sites, blots were incubated for 2 h in a Tris/NaCl buffer (10 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) containing 5% bovine serum albumin. A 1:1000 dilution of a rabbit anti-rat PAI-1 serum [29] (American Diagnostic, Greenwich CT, USA) was then added and the blots incubated for 4 h at room temperature. After 3 washes with Tris/NaCl buffer bands were visualised with 10 μl of a 1:500 dilution of peroxidase-conjugated goat anti-rabbit antibody. As a positive control for free PAI-1, dexamethasone-stimulated HTC rat hepatoma cell line-conditioned medium treated with 2-mercaptoethanol was used [30]. Plasma of endotoxin treated rats known to contain the PAI-1/t-PA complex not treated with mercaptoethanol was used a control for complexed PAI. [31]

Fibrin autography. This was performed according to the method of Granelli-Piperno and Reich. [32] After SDS-polyacrylamide gel electrophoresis, SDS was removed by washing the gel with water containing 2.5% Triton X-100, followed by a wash in a buffer containing 100 mmol/L NaCl, 50 mmol/L Tris, pH 7.7. The gel was then positioned on a fibrin indicator gel containing 30 μg/ml of human plasminogen, and incubated at 37° C in a humidified atmosphere. PA activity was detected as clear lysis

zones in an opaque background.

Statistics.
Data are expressed as mean ± standard deviation (SD) of triplicates. A significant difference between triplicates was defined as having a $P<0.05$ in a Student t-test.

RESULTS

PAI-1 and t-PA expression in vivo. To study the effect of circulating endotoxin on the expression of PAI-1 and t-PA in the vessel wall, total RNA was extracted from the medial layer of both rat and rabbit aortas, in basal conditions and after parenteral administration of endotoxin. PAI-1 mRNA was found in rat aorta tissue in basal conditions (fig. 1A, lane 1). Upon intraperitoneal injection with 50 μg/kg of endotoxin, a very strong increase in mRNA level was observed after 1 h, which at 3 h had already started to decrease. Low levels of t-PA mRNA were observed in rat aortic media in basal conditions and showed only little modulation 1 and 3 h after administration of endotoxin (fig. 1 A, lanes 2 and 3 respectively).
A similar effect was seen in the rabbit. PAI-1 mRNA in rabbit aorta was strongly induced 30 minutes after intravenous injection of endotoxin (1 μg/kg) (fig. 1 B, lanes 1 and 2 respectively). In the rabbit no t-PA mRNA could be detected in the aortic media in basal conditions, or after endotoxin treatment. This probe however, was able to hybridise with t-PA mRNA in other tissues such as myocardium and liver (fig. 1 B, lane 3 is myocardium, 30 minutes after 1 μg/kg endotoxin). These data suggest that vascular SMC can be potent producers of PAI-1 under certain circumstances such as

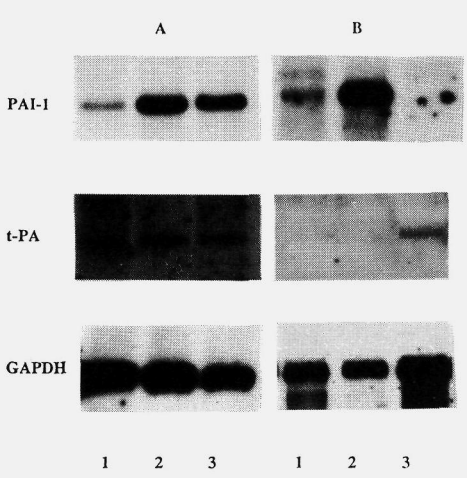


Figure 1. Northern blot analysis of 10 μg/lane of total RNA from the medial layer of rat and rabbit aorta before and after endotoxin injection, and hybridised with ^{32}P-labelled plasminogen activator inhibitor type-1 (PAI-1), tissue plasminogen activator (t-PA), and glyceraldehyde-3-phosphatedehydrogenase (GAPDH) cDNA fragments.
Panel A: Rat aortic media RNA, lane 1: 60 min af-ter vehicle injection, lane 2: 60 min after endotoxin injection (50 μg/kg intraperitoneally), lane 3: 180 min after endotoxin injection (50 μg/kg).
Panel B: Rabbit aortic media RNA, lane 1: 30 min after control injection (vehicle), lane 2: rabbit aorta 30 min after endotoxin injection (1 μg/kg intravenously), lane 3: rabbit myocardium, 30 min after endotoxin injection. The findings are representative of three independent experiments.

endotoxaemia and profoundly modulate the local fibrinolytic/proteolytic balance in or

near the vessel wall. Moreover, the PAI-1 induction in the aorta media by endotoxin is not species specific, in both species the PAI-1 mRNA is increased, whereas the t-PA expression remains low.

PAI activity in cultured rat aortic SMC. A strong induction of PAI-1 mRNA by endotoxin was found in the aortic media of both rat and rabbit, which consists almost solely of vascular smooth muscle cells. Subsequently we assessed the effect of endotoxin on PAI activity in cultured smooth muscle cell-conditioned medium to determine if endotoxin can directly affect the production of PAI-1 by cultured rat aorta smooth muscle cells, without the interference of other tissues such as endothelium and leukocytes as *in vivo*. PAI activity in the conditioned medium of serum free, quiescent cells was low. However, PAI activity in the conditioned medium rose from 20±6 IU/mg protein in serum free conditions to 283±16 IU/mg protein 4 h after addition of 10% (v/v) NCS and slowly started to decline thereafter (fig. 2). Treatment with 10 μg/ml endotoxin together with 10% NCS increased PAI activity in SMC-cell conditioned medium compared to control. The effect of endotoxin on PAI activity was maximal at 16 h, when PAI activity had risen from 152±20 IU / mg protein to 226±28 IU / mg protein, representing an increase of 50±7 % ($p < 0.01$, $n=4$) (fig.3). The presence of serum was an absolute requirement, as a wide range of doses of endotoxin (from 1 ng/ml to 50 μg/ml) did not have any effect in serum-free conditions. We

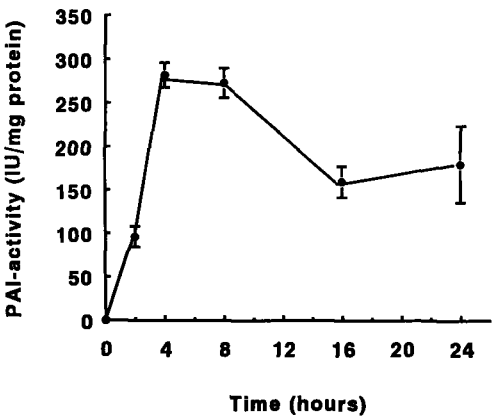


Figure 2. Accumulation of plasminogen activator inhibitor (PAI) activity in medium conditioned by cultured rat aortic smooth muscle cells. Confluent cultures were kept serum free for 48 h and then switched to *medium containing 10% newborn calf serum (NCS). At the specified timepoints the supernatant was collected for* PAI activity measurement and the cells harvested with 0.4 M NaOH for protein determination. PAI activity is expressed in equivalents of tissue plasminogen activator in international units (IU) per mg protein.
Each point represents the mean ± standard deviation (SD) of 4 experiments, performed in duplicate.

subsequently showed that the PAI activity in rat aortic SMC cell-conditioned medium could be attributed to type-1 plasminogen activator inhibitor.

Identification of PAI-1 and PAI-1/t-PA complex by immunoblotting. Immunoblotting with specific rabbit anti-rat PAI-1 antiserum was used to identify the detected PAI activity as PAI type I. After immunoblotting two bands could be detected in the rat aortic SMC cell conditioned medium (fig. 4). One band co-migrated with a band present in the 2-mercaptoethanol-treated conditioned medium of dexamethasone-stimulated HTC rat hepatoma cell line of 50 kD, corresponding to free PAI-1. [30] The second band co-migrated with a higher molecular weight band present in the serum of endotoxin treated rats known to contain large amounts of PAI-1/t-PA complex. [31] This identified the observed PAI activity as PAI type-1. Furthermore the PAI activity assay showed that (part of the) PAI-1 in the conditioned medium of rat aortic SMC is present in a free and active form.

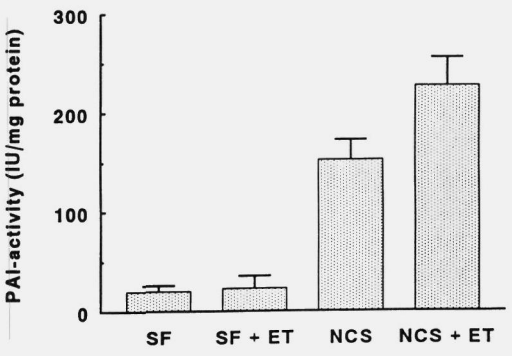


Figure 3. Plasminogen activator inhibitor (PAI) activity in medium conditioned by cultured rat aortic smooth muscle cells. Confluent cultures were kept serum free for 48 h and then switched to medium containing 10% NCS with or without 10 μg/ml endotoxin. The supernatant was collected after 16 h for PAI activity measurement and the cells were dissolved with 0.4 M NaOH for protein determination. PAI activity is expressed in equivalents of t-PA in international units (IU) per mg protein. Each point represents the mean $\pm$ SD of 4 experiments, performed in duplicate.

Plasminogen activator activity in cultured rat aortic SMC. Serum free rat aortic SMC cell-conditioned medium was analysed on fibrin zymography gel for the presence of plasminogen activators, either in free form or in complex with PAI. Only one area of lysis was observed, corresponding to PA/PAI complex. On the basis the identification of PAI-1 antigen (see above) and the fact that both PAI-1 and t-PA mRNA production in these cells is observed (see below), but not of u-PA, or of PAI-2, we rejected possibilities that this band is other than t-PA.PAI-1 complex. No lysis was observed in correspondence of the free t-PA (or u-PA) standard, this indicates that the concentration of PAI-1 is well in excess of PA under these conditions (fig.5). The fact that no active t-PA or u-PA could be detected in rat aortic SMC conditioned medium is in agreement with the determination of net PAI activity in serum free (18 h) cell conditioned medium.

PAI-1 gene expression in cultured rat aortic SMC. The increase in PAI-1 activity after addition of serum to quiescent rat aortic SMC cells suggests that PAI-1 mRNA is induced by serum factors, and that this can be further increased by endotoxin. We analysed total RNA extracted from rat aortic SMC cells on a Northern blot, probed with a ^{32}P-labelled PAI-1 cDNA probe. All rat aortic SMC cultures had been serum deprived for 48 h before the beginning of the experiments. Rat aortic SMC in a growth arrested serum free situation express very little PAI-1 mRNA. When cultured in the presence

Figure 4. Identification of plasminogen activator inhibitor type-1 (PAI-1) and PAI-1/PA (plasminogen activator) complex by immunoblotting.
Lane 1: 150μl of serum-free medium conditioned by rat aortic smooth muscle cells. PAI activity in the supernatant was 30.2 IU/ml. A single band of PA/PAI-1 complex is co-migrating with the control t-PA/PAI-1 complex in lane 2 in addition to a band of free PAI-1. Lane 2: control plasma of endotoxin treated rats containing both free PAI-1 and PAI-1/t-PA complex. [30] Lane 3: Medium conditioned by dexamethasone-stimulated HTC rat hepatoma cells (American Diagnostic, Greenwich, CT). [29] The conditioned medium was treated with 2-mercaptoethanol and therefore contained only free PAI-1.

of 10% NCS, they expressed large amounts of PAI-1 mRNA, with a maximum induction reached between 3 and 6 h (fig. 6). After treatment with 10 μg/ml endotoxin in the presence of 10% NCS no further induction of the PAI-1 mRNA could be observed.

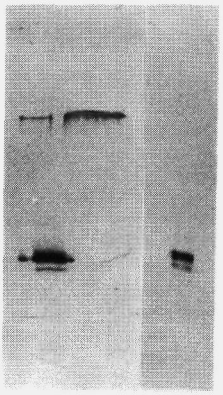


Figure 5. Fibrin autography, showing activity of free plasminogen activators and of PA-inhibitor complexes.
Lane 1: 50 μL of serum-free medium conditioned by rat aortic smooth muscle cells. A single area of lysis is observed in the region of the PAI-1/PA complex. Lane 2: rat urine containing both active urokinase (u-PA) and t-PA. Lane 3: human umbilical vein endothelial cell-conditioned medium known to contain PAI-1/PA complexes.

Expression of other genes of the fibrinolytic system in cultured rat aortic SMC. Both t-PA and the lipoprotein receptor-related protein (LRP) mRNA were present in very low quantities in serum free rat aortic SMC cultures. Upon addition of 10 % NCS a strong and fast increase of both t-PA and LRP mRNA was observed. The increase was maximal after three hours for t-PA and after one h for LRP and started to decline thereafter. Endotoxin (10 μg/ml) increased the mRNA level of LRP (fig.6). Also the t-PA mRNA level was increased by endotoxin, although not very profound. This is in contrast with our findings in animal experiments where endotoxin had no effect on the t-PA mRNA levels *in vivo* (fig.1).

We could not demonstrate expression of mRNA of the other genes involved in the fibrinolytic system: urokinase plasminogen activator, plasminogen activator type-2 and plasminogen (data not shown).

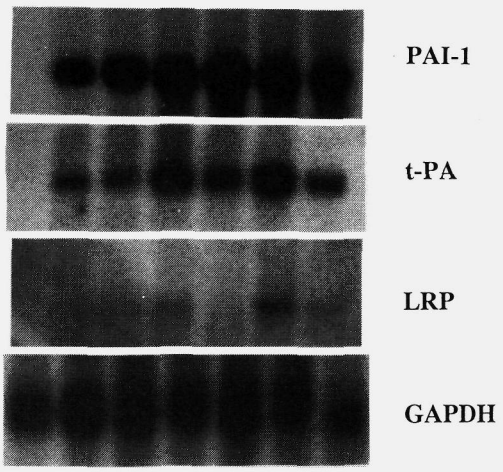
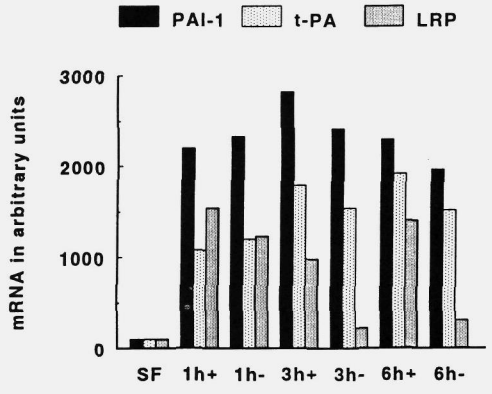


Figure 6A. Northern blot analysis of 5 μg/lane of total RNA extracted from cultured rat aortic smooth muscle cells one hour after adding serum free medium (SF) and 1, 3, and 6 h after adding medium supplied with 10 % (v/v) newborn calf serum without endotoxin(-) or with 10 μg/mL endotoxin (+). The same blot was hybridised with ^{32}P-labelled plasminogen activator inhibitor type-1 (PAI-1), tissue plasminogen activator (t-PA), Low density lipoprotein receptor-related protein (LRP) cDNA fragments and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Figure 6B. The densities of mRNA for each of the proteins PAI-1, t-PA and LRP are determined and normalised for the internal control GAPDH. The normalised mRNA densities are expressed in arbitrary units.



DISCUSSION

The administration of endotoxin to healthy volunteers profoundly affects the fibrinolytic balance in the circulation, and an increase in PAI activity that is preceded by an earlier increase in t-PA activity can be observed. [33] In human subjects with a severe gram-negative sepsis and resulting endotoxaemia a strong increase in circulating PAI-1 levels is observed. This increase in PAI-1 might contribute to clinical complications of sepsis such as disseminated intravascular coagulation (DIC) and persistently high levels of PAI-1 are correlated with bad outcome in these patients. [34] It is known that injection of small doses of endotoxin also causes a marked increase in plasma PAI activity in rats [31] and rabbits [16]. The source of this circulating PAI-1 is uncertain, but increase in PAI-1 mRNA in cultured endothelial cells after addition of endotoxin is well documented. [16-18] Very little is known however, about the role of the other main component of the vessel wall, smooth muscle. We therefore studied the effects of endotoxin on PAI-1 expression in smooth muscle cells both *in vivo* in animal experiments, and in cultured vascular smooth muscle cells.

PAI-1 mRNA is expressed in the rat thoracic aorta medial layer and a strong induction, approximately 6 fold, was observed rapidly after injection of endotoxin. An identical response of PAI-1 mRNA to endotoxin was observed in the rabbit aortic media, which showed that the reported effect is not specific for the rat. The rapid increase in PAI-1 production by the SMC might contribute to the intravascular coagulation problems occurring during severe endotoxaemia. We could demonstrate the presence of low levels of t-PA mRNA in the media of the aorta vessel wall in rat, whereas no t-PA mRNA could be detected in the rabbit aorta. This does not necessarily indicate a fundamental difference between these species, since in the rat the amount of mRNA was already close to the detection limit of the method, and in the rabbit it might have been just be under the detection limit. Administration of endotoxin had no effect on t-PA mRNA in the rat aorta media and although previously a decreased t-PA activity in aortic tissue extracts after endotoxin treatment was found, [21] but this was more likely due to increased production of PAI-1 and LRP than decreased production of t-PA.

The effect of endotoxin on the production of PAI-1 by smooth muscle cells in the aortic media may be a direct effect or a secondary effect, e.g. mediated by cell-cell contact with endothelium or monocytic cells, or through circulating factors produced by other cell types. For instance several tissues are known to increase nitric oxide (NO) levels in circulation after injection of endotoxin in rats. [35] To rule out interaction with other tissues, we have analysed the production of PAI-1 and t-PA in cultured isolated aortic media smooth muscle cells and the effect of endotoxin on this production. In conditioned medium of quiescent rat aorta smooth muscle cells PAI activity could be shown, and it was identified as PAI-1 by immunoblotting. PAI-1 was present both in a free form and in complex with plasminogen activator, as identified by immunoblotting

and fibrin autography, the free PAI-1 was at least in part active as shown in the PAI activity assay. Cultured rat aortic smooth muscle cells produced, in addition to PAI-1, a plasminogen activator that in the conditioned medium was present mainly in complex with PAI-1. Cultured rat aortic SMC express the mRNA for PAI-1, t-PA and LRP. Compared to the serum free situation PAI-1, t-PA and LRP mRNA levels in cultured rat aortic SMC were strongly increased after addition of serum. The net result of this was an increase in PAI activity in the cell-conditioned medium.

After addition of endotoxin together with serum to these cells, an increase of PAI activity in the conditioned medium of approximately 50% was observed. No effect on PAI-1 mRNA could be observed. This *in vitro* observation is in contrast with the endotoxin-induced increase in PAI-1 mRNA in vascular SMC in animal experiments and suggests that the increase in PAI-activity in the conditioned medium following addition of endotoxin is most likely due to post-transcriptional processes. However, it must be considered that differences in mRNA density of 50%, corresponding to the observed increase in PAI-activity are not easy to detect, and may have occurred unnoticed.

After addition of endotoxin together with serum a further increase in t-PA mRNA levels was observed in cultured SMC, this increase of 50-80% was consistent in 3 independent experiments. This is in contrast to what can be observed *in vivo* where endotoxin strongly increases PAI-1 mRNA without affecting t-PA mRNA levels. These data suggest that the effect of endotoxin on the production of PAI-1 and t-PA by smooth muscle cells *in vivo* is under a regulatory mechanism other than that in cultured SMC. *In vivo*, endotoxin might act indirectly in a way that involves other factors including cell-cell interactions with other cell types, such as endothelium or monocytic cells, or the production of circulating signal molecules. In serum-free conditions, no effect of endotoxin on the cultured rat aortic SMC could be observed. Recently it was shown that the CD14 protein is an endotoxin receptor; and although SMC are not able to synthesize endogenous CD14, they are able to bind serum-derived (soluble) CD14, upon which they are susceptible to endotoxin activation. [36]

The low density lipoprotein-receptor related protein/α_2-macroglobulin receptor (LRP), which has recently been reported to be produced by SMC is thought to be involved in the binding and subsequent intracellular degradation of t-PA and t-PA/PAI-1 complexes by SMC [10-13]. We therefore assessed the effect of serum and endotoxin on the production of LRP mRNA. Serum factors strongly increased the levels of LRP mRNA in cultured rat aortic SMC, and moreover endotoxin potentiated this increase. The rapid increase in LRP may have an effect on the net t-PA activity via the binding and subsequent degradation of t-PA by SMC. In fact we found that the increase in t-PA mRNA after addition of serum to cultured SMC was not followed by an increase in active t-PA or t-PA/PAI complex in the conditioned medium. This might be explained by the concomitant increase in PAI-1 that is effective in decreasing net t-PA activity and

moreover, the increase in LRP may enhance internalisation and subsequent degradation of both active t-PA and t-PA/PAI complex.

It is often stated that changes in the plasma fibrinolytic balance reflect a modulation of the rate of secretion and/or synthesis of fibrinolytic factors by the endothelium. This assumption is largely based on the fact that cultured human umbilical cord endothelial cells synthesise t-PA and PAI-1, and that the production of these factors can be modulated by several biologically active substances, such as tumor necrosis factor, [37] interleukin-1, [18,38] and endotoxin. [17,18] We found surprisingly high levels of PAI activity, approximately 50 IU/ 10^5 cells, 4 h after serum stimulation of serum-starved SMC. This amount is comparable to, or higher than what is observed with cultured human endothelial cells under similar conditions. [26,37] Although from experiments conducted *in vitro* it is not possible to draw any conclusion on the relative amounts of fibrinolytic factors produced *in vivo* by vascular endothelial and smooth muscle cells, it should be pointed out that for a given section of muscular resistance arteries, there are several layers of SMC underlying an endothelial cell monolayer, suggesting that the contribution of SMC to the local and even systemic fibrinolytic/proteolytic balance may be quantitatively important. Recently Lupu *et al* [39] and Schneiderman *et al* [40] showed by in situ hybridisation, expression of the PAI-1 gene in the media of human arteries, that could be contributed to both macrophage-like cells and mesenchymal cell assumed to be smooth muscle cells. This is in agreement with our finding that rat and rabbit aortic medial cells *in vivo* express the PAI-1 gene.

The finding that SMC are likely to modulate the fibrinolytic balance within the vessel wall has several potential implications. The lysis of a vessel wall thrombus is strongly influenced by the amount of plasminogen, plasminogen activators and their inhibitors that are incorporated in the thrombus. It is conceivable that fibrinolytic factors produced at the interface between the thrombus and the vessel wall could significantly alter the local fibrinolytic balance and thereby influence the susceptibility of the thrombus to lyse.

Besides its role in fibrinolysis plasminogen activation also plays a pivotal role in extracellular matrix degradation and cell surface-localised proteolysis. Plasmin is not only involved in digesting several extracellular matrix constituents like collagen type IV, [41] glycoproteins and proteoglycans, [42] but it can also activate latent procollagenases. [43] We have shown before that rat aortic SMC can degrade extracellular matrices produced by smooth muscle cells through activation of plasmin. [44] For these reasons modulation of plasminogen activation within the vessel wall is likely to affect cell migration through it. This may influence the infiltration of inflammatory or tumour cells, as well as the migration of SMC themselves from the media to the intima in the development of atherosclerotic lesions and after mechanical trauma. Preliminary data from experiments with transgenic mice support the role of plasminogen activation in cell migration through the vessel wall. Metastasis of Lewis lung carcinoma cells in PAI-

1 over-expressing mice was decreased, [45] and the rate of neointima formation in u-PA deficient mice was reduced. [46]

In conclusion; vascular smooth muscle cells play an important role in addition to endothelium cells in the regulation of the local fibrinolytic balance and possibly in the fibrinolytic/proteolytic balance in circulation too. Endotoxin increases PAI activity in circulation and is a potent stimulus for the production of PAI-1 mRNA in the aortic media, consisting mainly of smooth muscle cells, of rat and rabbit. Cultured aortic SMC on the other hand respond in a different way as the PAI-1 mRNA is hardly affected by endotoxin. The net result however, is the same, namely an increase in PAI-activity in the conditioned medium.

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CHAPTER 4.

PLATELET LYSATE AND PLATELET PRODUCTS INCREASE EXTRACELLULAR PROTEASES AND PROTEASE INHIBITORS IN CULTURED RAT VASCULAR SMOOTH MUSCLE CELLS.

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Submitted.

ABSTRACT.

In the damaged vessel wall, adhering platelets release several products that can activate the underlying smooth muscle cells (SMC). These cells start to migrate from the media to the intima; extracellular proteolysis is a requirement for this process.

We investigated the ability of platelet products to modulate extracellular proteolysis in cultured rat aortic smooth muscle cells (RASM).

The addition of platelet lysate (4. 10^6/mL) to quiescent cells resulted in an increase in plasminogen activator inhibitor (PAI) activity in the conditioned medium from 1.3 to 6.1 IU/mL. Platelet-derived growth factor (PDGF)-BB (1 nmol/L) and serotonin (0.1 mmol/L) increased PAI activity from 1.5 ± 0.1 to 6.4 ± 0.6 IU/mL and from 2.0 ± 0.4 to 5.3 ± 0.15 IU/mL respectively (mean $\pm$ SD, n=3). Northern blotting revealed a dose-related increase in mRNA of tissue plasminogen activator (t-PA) and its inhibitor PAI-1 after the addition of recombinant PDGF-BB. In addition mRNA for matrix metalloproteinase (MMP)-3 and the tissue inhibitor for metalloproteinases (TIMP-1) were increased after the addition of PDGF, LDL receptor-like protein (LRP) and MMP-2 mRNA were found constitutively expressed in RASM and could not be modulated by PDGF.

In conclusion: platelet factors directly modulate extracellular proteolysis and may thus contribute to the migration of SMC through the vessel wall in addition to their well-recognized role as a chemoattractant for SMC.

1. INTRODUCTION

Platelet adhesion to the vessel wall can cause lumen obstruction, activate the coagulation cascade and cause the occurrence of intravascular thrombi. Moreover, activated platelets release several mediators which target the smooth muscle cells (SMC), stimulating their migration and proliferation in the arterial intima. [1] Platelet products play a pivotal role in the pathogenesis of atherosclerotic lesions, in restenosis after angioplasty, in graft failure in coronary bypass surgery and vessel wall hypertrophy in hypertension. [2, 3, 4] The capacity of smooth muscle cells to synthesize DNA and divide is re-acquired through a shift from a quiescent, contractile type to a proliferative, synthesizing phenotype. [5, 6, 7] The synthesizing type SMC are responsive to platelet derived growth factor (PDGF) and express the PDGF-beta receptor subtype. [8, 9] The potential role of PDGF in stimulating vessel wall hypertrophy/hyperplasia has been demonstrated in a rat carotid artery balloon denudation model where infused PDGF was able to increase intimal hypertrophy. [10] Blocking antibodies against platelets [11] or against PDGF [2] were reported to attenuate this intimal thickening.

Extracellular proteolysis is necessary for smooth muscle cells to be able to migrate through the vessel wall by digesting the surrounding extracellular matrix. The two main pathways involved in extracellular proteolysis are the plasminogen activator pathway (reviews: [12, 13]). and the matrix metalloproteinase (MMP) system. [14] Rat aortic SMC are able to produce several components of the plasminogen system [15] : tissue-type plasminogen activator (t-PA), its main inhibitor plasminogen activator inhibitor type-I (PAI-1) as well as low-density lipoprotein receptor-like protein or α_2-macroglobulin receptor (LRP), the receptor involved in clearance of t-PA and t-PA/PAI complexes. SMC have the potential to convert the abundantly circulating protein plasminogen to the active protease plasmin. Plasmin is able to degrade several matrix constituents such as collagen types IV and V, proteoglycans, laminin and fibronectin [16], but plasmin is also able to activate latent matrix metallo-proteinases. [17] The activated metalloproteinases, labelled MMP-1 through -10 can degrade virtually all extracellular components of connective tissues [14], their activity is regulated by tissue inhibitors of metalloproteinases (TIMP) and by α_2-macroglobulin. [18, 19] Vascular smooth muscle cells can produce, in addition to the components of the plasminogen pathway, several metalloproteinases such as : MMP-1, [17, 20] MMP-2, [21, 22, 23] MMP-3, [20, 24] MMP-9, [20, 25, 26] as well as the inhibitors TIMP-1 and TIMP-2 [20, 26] in addition to the aforementioned components of the plasminogen system.
In this paper we have established the effect of platelet lysate as well as of the platelet products PDGF and serotonin on the production and activity of extracellular proteinases of the plasminogen system and the metalloproteinase system in cultured rat aortic smooth muscle cells.

2. METHODS AND MATERIALS

2.1. Cell culture

Rat aortic smooth muscle cells (SMC) were obtained from male Sprague-Dawley rats by enzymatic dispersion and cultured as described by Sperti *et al.* [27] The cells used in the experiments (passages 5 to 20) were dissociated with trypsin and seeded at a density of 7-10. 10^3 cells/cm^2 in 24 multiwell dishes in DME medium supplied with 10% newborn calf serum. Confluent cultures were left in serum free (SF) medium for 48 hours. At time point zero the cells were rinsed twice and fresh SF medium with the specified substances was added to the cultures. The medium was sampled at the specified time intervals and aliquots snap frozen in liquid nitrogen. For gene induction experiments, cells were cultured in a similar way in 60 mm dishes.

2.2. Platelet preparations

Fresh platelets from healthy volunteers were isolated from 50 mL blood with 1/10 volume of 0.11 mol/L citrate. Platelet-rich plasma was separated from the blood cells by centrifugation (120 x g. for 20 min. at 4°C). Platelets were spun down from this plasma (2500 x g. for 20 min., 4°C), resuspended in phosphate-buffered saline (pH 7.35) containing 2 mg/ml glucose and completely lysed by three freeze-thaw cycles. This platelet lysate was stored in small aliquots at -70°C.

2.3. PAI activity Assay

Plasminogen activator activity and plasminogen activator inhibitor activity in the cell-conditioned medium were determined as previously described. [28] PA activity was measured in the presence of CNBr digested fibrin fragments and plasminogen. PAI activity was assessed by titration with known amounts of two-chain t-PA in the presence of CNBr digested fibrin fragments and plasminogen. In both cases, plasmin generation was assessed by monitoring at different time points the cleavage of the chromogenic substrate D-Val-Leu-Lys-pNA in a spectrophotometer at a wavelength of 405 nm.

2.4. Gelatine gel zymography

Samples of cell extracts (in 0.5% Triton/PBS) or conditioned medium are separated at 4°C on a non-denaturing 0.1% SDS, 8% polyacrylamide mini-gel with 0.2% gelatine copolymerized (porcine skin gelatine). This is an adaptation of the method described by Heussen and Dowdle. [29] After electrophoresis the SDS is washed away with 2.5% Triton X-100 and the gel is rinsed with and incubated in an incubation buffer (50 mM Tris pH 8.0, 5 mM $CaCl_2$, 1 mM $ZnCl_2$) at 37 °C for 18 hours, during this time the proteinases digest a clear lysis zone in the gelatine background which is visualized after staining with Coomassie Blue. Gelatine is degraded by MMP-2 and -9 also to some extent by MMP-3 and MMP-1. [30]

The pro-MMPs are partially activated by SDS and can be distinguished in these gelatine zymograms from activated MMP's by their 10 kD higher molecular weight.

2.5. Fibrin overlay zymography

This was performed according to the method of Granelli-Piperno and Reich. [31] Sodium dodecyl-sulphate polyacrylamide electrophoresis was performed using a 10% (w/v) polyacrylamide in 0.1 % (w/v) SDS, 0.38 mol/L Tris, pH 8.8, with a 5% polyacrylamide stacking gel in 0.1 % SDS, 0.12 mol/L Tris, pH 6.8. After electrophoresis, SDS was removed by washing the gel with water containing 2.5% (v/v) Triton X-100, followed by a wash in a buffer containing 100 mmol/L NaCl, 50 mmol/L Tris, pH 7.7. The gel then was layered onto a fibrin indicator gel containing 30 μg/mL of human plasminogen, and incubated at 37°C in a humidified atmosphere.

2.6. RNA extraction and analysis

RNA was extracted according to Chomczinski and Sacchi. [32] Ten microgram of total RNA per lane was run on an 0.8% agarose and 8% formaldehyde gel according to Sambrook et al. [33] and transferred (Vacugene System, Pharmacia) onto a nylon membrane (Hybond N, Amersham International). The blot was hybridized with a random primer ^{32}P-dCTP- labelled probe (Amersham Multiprime Labelling Kit) in 0.5 mol/L sodium phosphate, 7% (w/v) SDS at 60 °C. Autoradiography was performed on Kodak X-AR film with intensifying screens. The films were digitally scanned and the scanned values for mRNA intensities were normalised for the scan reading of the internal standard glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as described before. [34] Ratios were expressed in arbitrary dimensionless units.

2.7. DNA fragments used for hybridisation

The PAI-1 probe was a 1.1 kB *Pst I* fragment of a human PAI-1 cDNA with a high homology to the rat PAI-1 gene. [35] The t-PA probe was a 2.0 kB *Bgl II* fragment from the human t-PA cDNA. [36] A 1.2 kB *Pst I* fragment from the rat GAPDH cDNA was used as an internal standard. [37] The LRP probe was a 6.0 kB *XhoI-EcoRI* fragment of human cDNA, [38], the MMP-1 probe was a 1.5 kB *Xba I* fragment from collagenase-I cDNA. [39] The MMP-2 probe was a 1.2 kB EcoRI fragment of the human cDNA, [40] the MMP-3, MMP-9 and TIMP-1 probes were cDNA fragments obtained by reverse transcriptase PCR, kindly provided by Dr. Hanemaaier. [30]

2.8. Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (SD) from the mean. Differences between experimental conditions were assessed by the Student t-test. A value of $P < 0.05$ was considered significant.

2.9. Materials

Recombinant PDGF-BB and PDGF-AA were obtained from Peninsula Laboratories Europe, St.Helens, UK. The chromogenic plasmin substrate p-nitroanilide-conjugated tripeptide (D-Val-Leu-Lys-pNA) and recombinant t-PA standard were purchased from Kabi-Chromogenix, Mölndal, Sweden. Plasminogen was purchased from Kordia-Eurostas, Leiden, the Netherlands. ^{3}H-labelled thymidine was obtained from Amersham International, Amersham, UK.

3. RESULTS

3.1. Increase in PAI activity in RASM-conditioned medium by platelet lysate, PDGF and serotonin.

Several stimuli can modulate the secretion of plasminogen activator inhibitor-1 and (tissue-type) plasminogen activator into the conditioned medium by cultured SMC. [34, 41] The effect of platelet lysate on the activities of plasminogen activators and plasminogen activator inhibitors in rat aortic smooth muscle cell-conditioned medium was determined 16 hours after the addition of platelet lysate. No PA activity could be demonstrated using the chromogenic substrate D-Val-Leu-Lys-pNA, but PAI activity in the RASM-conditioned medium was increased from 1.3 ± 0.4 to 6.1 ± 0.6 (n=3) IU/mL by platelet lysate in a dose-dependent fashion (Fig.1). To exclude the possibility that this PAI activity was originating from the platelets instead of being newly excreted by the SMC, we measured PAI activity in platelet lysates 16 hours after incubation at 37°C. PAI activity in the platelet lysates ($5 .10^8$/mL) decreased from 7.1 ± 0.6 to less than 0.1 IU/mL after 16 hours. We concluded that the residual activity from the platelets therefore does not significantly contribute to the PAI activity measured after 16 hours in the RASM-conditioned medium. In addition to the platelet lysate, we also assessed the effect of several purified platelet products on PAI activity in RASM-conditioned medium. The addition of recombinant PDGF-BB (1 nmol/L) to quiescent RASM cultures resulted in an increase in PAI activity after 16 hours in the RASM-conditioned medium from 1.5 ± 0.1 to 6.4 ± 0.6 IU/mL (mean$\pm$SD; $P < 0.01$, n=3, Fig.2). This increase was dose-related and the ED_{50} was estimated at 0.5 nmol/L PDGF-BB (Fig.3).

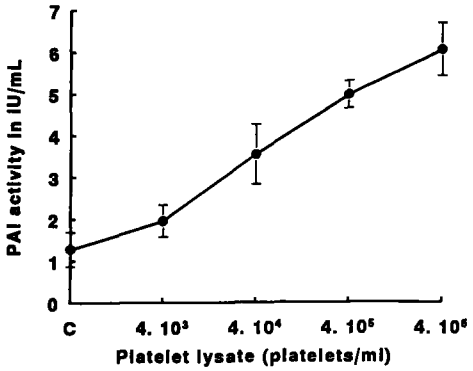


Figure 1.
Graph showing the increase in PAI activity in the conditioned medium of cultured rat aorta smooth muscle cells 16 hours after the addition of platelet lysate. The PAI activity is expressed in equivalents of IU/mL t-PA (mean $\pm$ SD, n=3).
C=control without platelet lysate. The contribution to the total PAI activity of PAI originating from the platelet lysate was found negligible after 16 hours incubation at 37 °C.

PDGF-BB and PDGF-AA are acting through different receptors (the ß- and the α-receptor respectively) and we compared the potency of the two recombinant PDGF homodimers to increase PAI activity in RASM-conditioned medium 6 hours after the addition of either PDGF-BB (1 nmol/L) or PDGF-AA (10 nmol/L) to quiescent

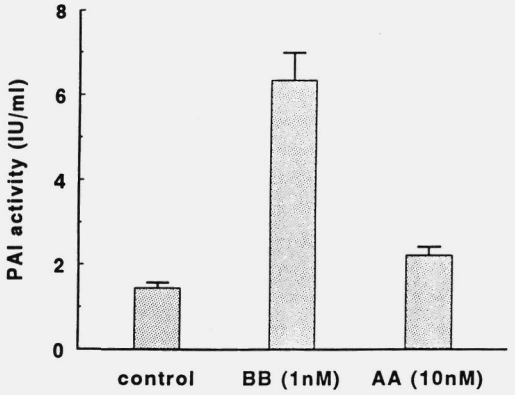


Figure 2.
Graph showing the PAI activity in the conditioned medium of cultured rat aorta smooth muscle cells after the addition of serum free medium (control) and medium with recombinant platelet-derived growth factor-BB (column BB, 1 nmol/L) or PDGF-AA (AA, 10 nmol/L). The PAI activity is expressed in equivalents of IU/mL t-PA (mean±SD, n=3).

cultures. PDGF-BB was much more potent (336±45% increase over control, n=3) than PDGF-AA (51±13% increase over control, n=3) (Fig.2). We conclude that platelet PDGF which is mainly of the heterodimer AB type will predominantly stimulate the PDGF ß-receptor.

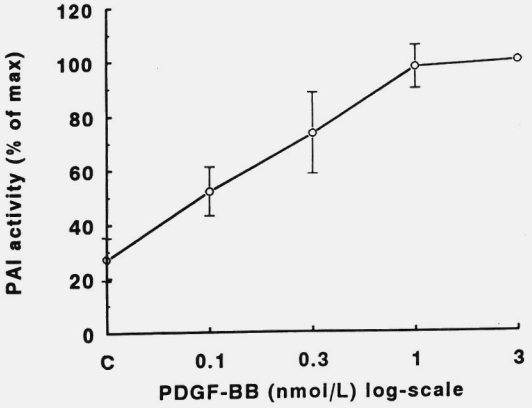


Figure 3.
Graph showing the dose-dependent increase in PAI activity in serum free cell-conditioned medium after the addition of platelet-derived growth factor (PDGF-BB, in nmol/L) in cultured rat aortic smooth muscle cells.
The PAI activity is expressed as a percentage of the maximum value reached at 3 nmol/L PDGF-BB (mean±SD, n=3).

Serotonin (5-HT, 0.1 mmol/L), another platelet product, was able to increase PAI activity in RASM-conditioned medium from 2.0±0.4 to 6.1±0.8 (Mean±SEM, n=4) after 6 hours. The increase was time-dependent with a maximum PAI activity reached around 6 hours after the addition of serotonin (Fig.4).

3.2. PDGF increases t-PA/PAI-1 complex in RASM-CM.

Plasminogen activators can be identified on a fibrin overlay zymography gel. [31] PAI-1/t-PA complex can be identified by fibrin zymography as well, because the complexed t-PA is partially re-activated by SDS. The rat aortic smooth muscle cell-conditioned medium was analysed on a fibrin zymography gel after the addition of PDGF to the cells. The amount of t-PA/PAI-1 complex in serum-free RASM-conditioned medium 8 and 24 h after the addition of PDGF BB (1 nmol/L), is increased compared to control

cultures.

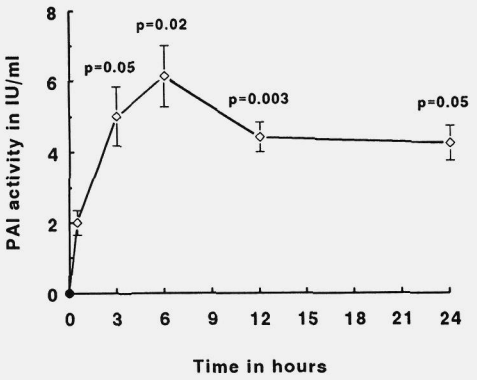


Figure 4.
Graph showing the PAI activity in the cell-conditioned medium of cultured rat aortic smooth muscle cells 0.5, 3, 6, 12 and 24 hours after the addition of the platelet product serotonin (5-hydroxy-tryptamine, 0.1 mM). Confluent cultures were kept serum-free for 48 hours before addition of serotonin. The PAI activity is expressed in equivalents of IU/mL t-PA (mean ± SEM, n=4).

Some free t-PA can be demonstrated after electrophoretic separation from the inhibitor PAI-1, and the amount of free t-PA is slightly reduced after the addition of PDGF (Fig.5). Taken together, the effect of addition of PDGF-BB is an increase in PAI activity and t-PA/PAI complex in RASM-conditioned medium, and a decrease in free or uncomplexed t-PA.

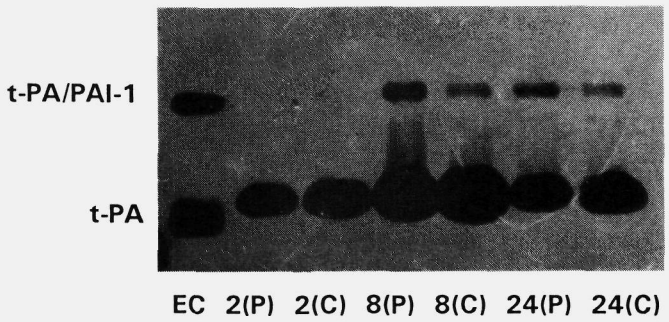


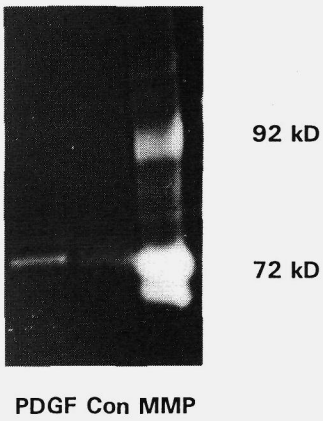
Figure 5.
Fibrin overlay zymography showing the increase in t-PA/PAI complex in RASM-conditioned medium, 8 and 24 hours after the addition of 1 nM PDGF-BB. The first lane (EC) contains human umbilical artery endothelial cell-conditioned medium known to contain both free t-PA and t-PA/PAI-1 complex. The other lanes contain RASM-conditioned medium 2, 8, and 24 hours after the addition of serum free control medium (C) or medium with 1 nmol/L PDGF-BB (P).

3.3. Effect of PDGF on the activity of metalloproteinases.

In the conditioned medium of rat aorta SMC, only the metalloproteinase MMP-2 can be shown (Fig.6). Both the pro-enzyme and the 10 kD shorter activated form (the main band) are visible. The rat protein has a slightly higher apparent molecular weight than the (human) control MMP-2 forms. After incubation with PDGF-BB (1 nmol/L) an increase in the amount of MMP-2 is observed. No activity of other metalloproteinases could be demonstrated. In the cell-associated fraction, extracted with 0.5% Triton-

X100, no gelatinase activity could be demonstrated (data not shown), suggesting that most of the MMP-2 protein is released in the conditioned medium.

Figure 6.
A gelatinase zymography gel, consisting of a poly acrylamide gel containing 0.2% porcine skin gelatine, showing activity of gelatinases (matrix metalloproteinase type-2) in rat aortic smooth muscle (RASM) cell-conditioned medium. Confluent cultures were kept serum free for 48 hours before the start of the experiments. The right lane (MMP) contains a purified MMP-2 and MMP-9 mixture. The two other lanes contain 16 hours cell-conditioned medium of RASM with serum free control medium (Con) and 16 hours after the addition of recombinant PDGF-BB (PDGF, 1 nmol/L).



3.4. Effects of PDGF on mRNAs of extracellular proteases and protease inhibitors.
Both tissue-type plasminogen activator and plasminogen activator inhibitor-1 mRNA can be shown in quiescent RASM. PDGF-BB increases the mRNA levels for t-PA and PAI-1 in a dose-dependent fashion (Fig.7). The increase is noticeable after 2 hours (Fig.8). The amount of mRNA for LDL-receptor related protein (LRP) is not significantly modulated by PDGF (Fig.7,8). Urokinase type plasminogen activator, plasminogen and plasminogen activator inhibitor type-2 mRNA could not be demonstrated in these cells, whether in quiescent RASM cultures, or after addition of PDGF (data not shown).
The mRNA of matrix metalloproteinase-2 can be shown in quiescent RASM cultures. No significant modulation of this MMP-2 mRNA by PDGF-BB could be observed (Fig.7,8). The mRNA levels of the tissue inhibitor of metallo proteinases (TIMP-1) was found to be strongly increased after addition of PDGF-BB (Fig.8). MMP-9 mRNA could not be demonstrated in these cells, whether in quiescent RASM cultures, or after addition of PDGF (data not shown). MMP-3 mRNA (stromelysin or transin-1) was very low in quiescent RASM cultures, but after the addition of PDGF-BB the mRNA increased in a dose-dependent fashion (Fig.7). The increase was already noticeable after 2 hours, and reached a maximum around 4 hours after the addition of PDGF (Fig.8).

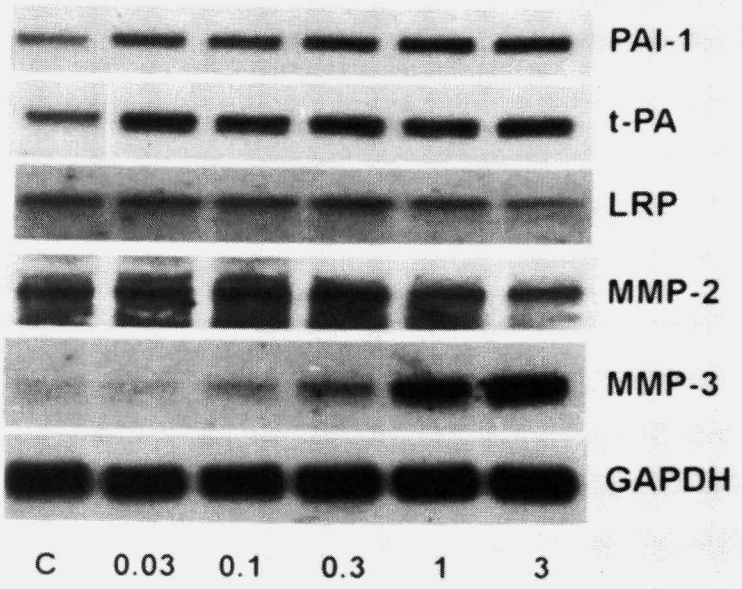
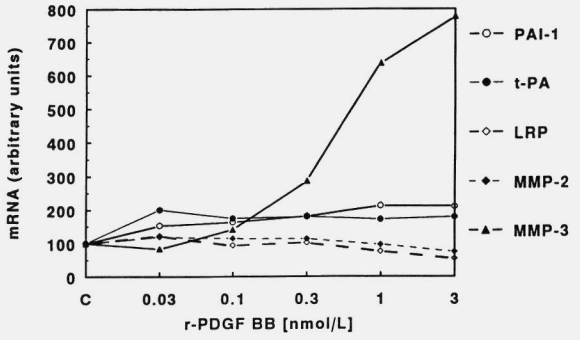


Figure 7.

(A, left) Northern blot showing the expression of PAI-1, t-PA, LRP, MMP-2, MMP-3 mRNA and the internal control GAPDH at increasing concentrations of PDGF-BB (in nmol/L). RNA is extracted 7 hours after the addition of PDGF to confluent cultures of RASM and 5 μg of total RNA is applied per lane.

(B, right) Graph showing the scanned mRNA densities from this Northern blot, normalised for the internal control GAPDH.



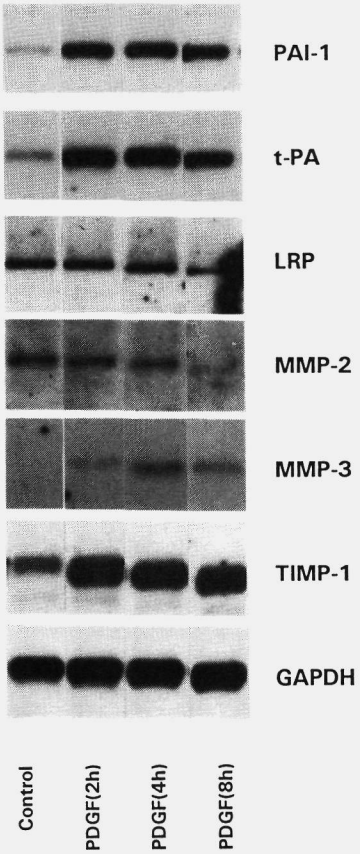
4. DISCUSSION

There is a large amount of experimental, pathological and clinical evidence supporting the involvement of platelets and of the biologically active substances released by them after adhesion, in the pathogenesis of the atherosclerotic lesions in the arterial wall. [1, 42]

Friedman *et al* [43] showed that formation of neointima after vascular injury was

markedly reduced in rats with prolonged thrombocytopenia, and concluded that platelet products stimulate SMC replication. On the contrary, Fingerle *et al.* [8] found that merely the migration of medial SMC is enhanced by chemotactic platelet products but replication in itself is not or hardly affected. Jawien *et al.* [10] concluded the same using infused PDGF-BB in a rat with mildly damaged endothelium. It is however not easy to demonstrate that the occurrence of new cells in the intima is due to either migration to, or proliferation within the intima, especially since both processes may take place at the same time *in vivo*. So, while there is substantial agreement on the chemotactic effect of platelet products on vascular SMC, very little is known on the modulation of extracellular proteolysis which necessarily takes place in migrating cells.

Co-ordinate and consecutive activation of plasminogen activators, plasmin and metalloproteinases is well documented during angiogenesis [12, 14], tumour cell



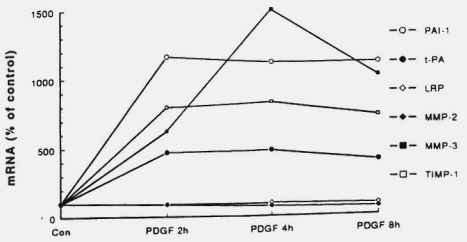


Figure 8.

(A, left) Northern blot showing the expression of PAI-1, t-PA, LRP, MMP-2, MMP-3, TIMP-1 mRNA and the internal control GAPDH at the specified times after addition of recombinant platelet-derived growth factor-BB. RNA is extracted 2, 4 and 8 hours after the addition of 1 nmol/L of PDGF-BB to (48 hours serum free) confluent cultures of RASM and 5 μg of total RNA is applied per lane.

(B, above) Graph showing the scanned mRNA densities from this Northern blot, normalised for the internal control GAPDH.

invasion and metastasis, [44, 45, 46] but appears to be important in the development of atherosclerosis as well. [11, 20, 25, 47] The significance of metalloproteinases for the migration of smooth muscle cells is further indicated by the fact that the MMP inhibitor GM 6001 was able to prevent migration of SMC completely in the rat carotic artery injury model. [47] Recently Ye *et al.* [48] reported a link between the genetic variation in the stromelysin (MMP-3) promoter region and progression of atherosclerotic disease.

We have shown before that rat aortic SMC are able to produce tissue plasminogen activator, its inhibitor PAI-1 and the receptor LRP involved in clearance of t-PA and t-PA/PAI complexes. t-PA can activate the abundantly circulating protein plasminogen to yield plasmin. Plasmin is able to degrade several matrix constituents, [16] but is also able to activate latent metalloproteinases, [17, 45] that can degrade virtually all extracellular components of connective tissues. We treated cultured RASM with platelet lysate to assess whether the production of fibrinolytic proteins was modulated and report a 5-fold increase in PAI activity, but not of t-PA activity in RASM-conditioned medium.

We have extended these observations assessing the effect of platelet factors, and in particular of PDGF and serotonin, on extracellular proteolysis in cultured smooth muscle cells. We found that PDGF-BB (1 nmol/L) and serotonin (0.1 mmol/L) were both able to increase PAI activity in the RASM-conditioned medium about 5 times. PDGF was shown to increase PAI protein in bovine SMC-conditioned medium 2 times, with an ED50 of 1.5 ng/ml=50 pmol/L. [41] These results are comparable to our findings in RASM, although we found a slightly higher ED_{50} of 200-500 pmol/L. Cultured SMC are reported to express both PDGF type-α and PDGF-ß receptors, [49, 50] and in our experiments recombinant PDGF-BB was much more potent (50-100 times) than PDGF-AA in increasing PAI activity in RASM-conditioned culture medium.

On the mRNA level, the effect of PDGF-BB is an moderate increase in both PAI-1 and t-PA, the resulting net effect on the protein level is an increase in the PAI-activity and the amount of t-PA/PAI complex in the cell-conditioned medium.

The production of the precursor of tissue collagenase/matrix metalloproteinase 1 (proMMP-1) by cultured human aortic medial smooth muscle cells (SMC) is reported to be significantly enhanced by the treatment of the cells with PDGF. [51, 52] We assessed the effect of PDGF on several other MMP's. Our results indicated that matrix metalloproteinase 2 was constitutively expressed in RASM and that the mRNA levels were not significantly modulated by PDGF. Both MMP-2 and proMMP-2 activity in RASM-conditioned medium could be shown on a zymogram, both in control cells and after addition of PDGF to the cells. The total amount of MMP-2 protein on the zymogram appears to be increased after addition of PDGF; this finding is not matched by a corresponding increase in MMP-2 mRNA, which was if anything (not significantly) decreased at the highest concentrations and incubation times with PDGF. This suggests that an other mechanism is responsible for the increase in MMP-2 protein on the zymogram. A possible explanation is that pro-MMP 2 is activated by MMP-3

after PDGF stimulation and that the activated MMP-2 is more efficient in digesting the indicator gel.

Matrix metalloproteinase 3 (or transin, homologous to the human stromelysin) mRNA was present in low amounts in quiescent SMC, but after the addition of PDGF it was strongly increased, in contrast to MMP-2. Diaz-Meco *et al.* [53] reported a PDGF-responsive element in the stromelysin promoter region, which suggests a direct induction of MMP-3 by PDGF, independent of the protein kinase-C system. We could not demonstrate MMP-3 by gelatin zymography, but this could be due to the low efficacy of MMP-3 in digesting the gelatine and does not necessarily mean that the mRNA is not translated into a functional protein.

We could not demonstrate any MMP-9 mRNA in the cultured rat aortic SMC, in agreement with the absence of MMP-9 activity on the gelatine zymogram of RASM-conditioned medium. However, MMP-9 mRNA was reported to be expressed in injured rat arteries, [47] in early cultures of rabbit aorta SMC [25] and after treatment with phorbol ester in baboon aortic SMC. [20]

In conclusion: platelet factors increase proliferation in cultured vascular smooth muscle cells from several species. PDGF increases the mRNA's for several extracellular proteolytic enzymes (t-PA, MMP-1, MMP-3) and their inhibitors (PAI-1, TIMP-1). An increase in (focal) proteolytic activity is a key factor in the onset of cell migration, and a concommitant increase in the inhibitors is necessary to prevent uncontrolled proteolysis and damage to the surrounding tissue. Nearly all the circulating PDGF is secured in the platelet alpha granules and therefore adhering and degranulating platelets are virtually the only source of PDGF from the circulation. A better knowledge of the molecular mechanisms induced by platelet products to promote cell migration might lead to therapeutic strategies effective in preventing the formation of vascular lesions.

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CHAPTER 5.

ANGIOTENSIN II INCREASES PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 AND TISSUE-TYPE PLASMINOGEN ACTIVATOR MESSENGER RNA IN CULTURED RAT AORTIC SMOOTH MUSCLE CELLS

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ABSTRACT

Background The role of angiotensin as a vasoconstrictor is well established. Lately, several other actions of this hormone on vascular smooth muscle (VSM) cells have been recognized including the induction of hypertrophy and/or DNA synthesis. Platelet derived growth factor (PDGF), a mitogen recently shown to increase plasminogen activator type 1 (PAI-1) synthesis in VSM cells, shares with angiotensin II (Ang II) several steps of its intracellular signaling pathway.

Methods and Results The expression of PAI-1 and tissue-type plasminogen activator (t-PA) mRNA in cultured rat VSM cells was studied. Northern blot analysis demonstrated a several-fold increase in the PAI-1 mRNA 3 to 8 hours after stimulation with 300 nmol/L Ang II. A similar response for t-PA mRNA was observed. This induction did not require the synthesis of an intermediate protein or peptide because it was not affected by cycloheximide. In the cell-conditioned supernatant, the net result was an increase in PAI-1 activity from 4.2 ± 1.8 to 13.2 ± 6.8 IU/mL 6 hours after the addition of 300 nmol/L Ang II (mean $\pm$ SD, $P \leq .008$, $n=6$). The Ang II-induced increase in PAI activity was dose-related, with a maximal effect at a concentration of 23 nmol/L ($n=3$), and an ED_{50} of 3.3 ± 1.5 nmol/L, ($n=3$). [Sar^1-Ile^8]angiotensin II, a specific competitive antagonist of Ang II, blocked 90 $\pm$ 9% ($n=3$) of the PAI activity induced by 10 nmol/L Ang II.

In basal conditions, fibrin overlay zymography demonstrated the presence of free t-PA. After stimulation with Ang II, lysis due to the in situ dissociation of t-PA was also present in the region of the t-PA/PAI-1 complex.

Angiotensin I (Ang I) elicited an increase in PAI activity similar to that obtained with equivalent doses of Ang II. Captopril (5μg/mL), an inhibitor of the angiotensin-converting enzyme (ACE), completely prevented the Ang I effect, demonstrating that VSM cells display an ACE-like activity.

Conclusions Recent research has demonstrated the existence of a localized vascular renin-angiotensin system. The finding that Ang II can potentially modulate the plasminogen activation in the arterial wall has important biological and therapeutical implications for the evolution of arterial wall thrombi and the migration of cells through the vessel wall in the genesis of atherosclerotic lesions. We speculate that the reduction in thrombotic events observed in patients with a previous myocardial infarction and in high-renin hypertensive patients treated with ACE inhibitors could be due at least in part to the decreased production of PAI-1 by VSM cells caused by these agents.

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Key Words: PAI-1, t-PA, angiotensin, smooth muscle.

INTRODUCTION

The renin-angiotensin system (RAS) plays a major role in vascular homeostasis. The physiological actions of angiotensin II (Ang II), the final common product of this pathway, of directly regulating vascular resistance and fluid and electrolyte balance through the modulation of aldosterone secretion have long been recognized[1]. In recent years, two major findings have added new interest to the study of the RAS: 1) the description of several other aspects of Ang II and in particular its possible role as growth factor and/or "hypertrophy" hormone for vascular wall myocytes[2-7], and 2) the demonstration of the existence of a local vascular RAS[1].

Several clinical observations have linked the RAS to an increased risk of thrombotic diseases. Angiotensin-converting enzyme (ACE) inhibitors can reduce the risk of a second coronary event in patients with heart failure caused by a previous myocardial infarction[8], and patients with "high renin" hypertension appear to sustain a higher risk of myocardial infarction compared with the "low renin" ones[9]. Accordingly, a genetically determined high activity of the ACE as observed in the presence of the DD genotype, is associated with a higher risk of myocardial infarction[10]. In vitro, a link between Ang II and the fibrinolytic system has been demonstrated, as Ang II increases plasminogen activator inhibitor type 1 (PAI-1) mRNA and protein in cultured rat astrocytes[11,12]. Recently, we and others demonstrated that vascular smooth muscle (VSM) cells both in culture and in vivo can synthesize tissue-type plasminogen activator (t-PA) and its specific inhibitor PAI-1 [13-15]. Interestingly PAI-1 mRNA expression has recently been reported to be higher in the thickened media of atherosclerotic arteries compared with normal vessels.[16] In cultured VSM cells, the synthesis of PAI-1 can be modulated by substances such as thrombin[17] and platelet derived growth factor (PDGF),[14,18] which share with Ang II several steps of its intracellular signaling pathway, namely the generation of inositol triphosphate, diacyl-glycerol [17,19] and the increase in intracellular Ca^{2+} [17,20,21]. Finally, infusion of Ang II in healthy volunteers substantially increases the level of PAI-1 antigen in the bloodstream[22]. Taken together these observations led us to formulate the hypothesis that Ang II could modulate the production of fibrinolytic factors in VSM cells.

In this report, we demonstrate for the first time that Ang II modulates t-PA and PAI-1 mRNA and activity in a cultured VSM cell system. If confirmed in vivo, these findings could have important implications for the lysis of arterial wall thrombi and for the migration of VSM cells from the media to the intima.

METHODS

Materials

Captopril, angiotensin I (Ang I), Ang II, and the angiotensin receptor antagonist, [Sar^1-Ile^8]angiotensin II were purchased from Sigma. Recombinant PDGF BB was obtained from Peninsula Laboratories Europe. The chromogenic plasmin substrate p-nitroanilide-conjugated tripeptide (Val-Leu-Lys-pNA) were purchased from Kabi. Plasminogen was purchased from Eurostas. ^{3}H-thymidine and ^{3}H-aminoacid mixture were from Amersham International, Amersham, UK. t-PA standard was obtained from Kabi.

Cell culture

Rat aortic VSM cells were obtained from male Sprague-Dawley rats by enzymatic dispersion according to Gunther et al.[23] and cultured as described by Sperti et al.[24]. The cells used in the experiments (passages 5 to 20) were dissociated with trypsin from T75 stock flasks and seeded at a density of 7000 to 10000 cells/cm2 in 24 multiwell dishes. Cells were grown to confluence in DME medium supplied with 10% newborn calf serum (4 to 6 days), rinsed twice and left in serum free (SF) DME medium for a further 48 hours. The cells were then rinsed twice and fresh SF medium with or without the specified drugs was added to the cultures. The medium was sampled at the specified time intervals and aliquots snap frozen in liquid nitrogen. For gene induction experiments, cells were cultured in a similar way in 60 mm dishes. In some experiments the protein synthesis inhibitor cycloheximide (10 μg/mL) was added 30 minutes before addition of Ang II. Preliminary experiments showed that this concentration of cycloheximide completely abolished Ang II-induced ^{3}H-labeled aminoacid incorporation.

PAI activity Assay

PAI activity in the cell-conditioned medium was determined as previously described[25]. A standard curve was obtained with known amounts of two-chain standard t-PA. PAI activity was assessed by titration with known amounts of two-chain t-PA in the presence of CNBr digested fibrin fragments and plasminogen. In both cases, plasmin generation was assessed by monitoring at different time points the cleavage of the chromogenic substrate Val-Leu-Lys-pNA in a spectrophotometer at a wavelength of 405 nm.

Fibrin overlay zymography

This was performed according to the method of Granelli-Piperno and Reich[26]. SDS-PAGE was performed using 10% polyacrylamide and 0.1 % SDS in 0.383 mol/L Tris, pH 8.8, with a stacking gel containing 5% polyacrylamide, 0.124 mol/L Tris, pH 6.8. After electrophoresis, SDS was removed by washing the gel with water containing

2.5% Triton X-100, followed by a wash in a buffer containing 100 mmol/L NaCl, 50 mmol/L Tris, pH 7.7. The gel then was layered onto a fibrin indicator gel containing 30 μg/mL of human plasminogen, and incubated at 37°C in a humidified atmosphere.

^{3}H-thymidine and ^{3}H-aminoacid incorporation

Preconfluent cultures in 24-multiwell dishes were kept SF for 48 hours. At time 0, after two washes with SF medium, 0.5 mL of SF medium with or without the specified drugs was added to each well. After 20 hours, the cultures were pulsed for 4 hours by adding 0.5 μCi per well of ^{3}H-thymidine. The experiment was terminated by washing twice with 0.9% NaCl, once with 0.5 mL per well of 10% trichloroacetic acid and twice again with saline. The cells were then dissolved in 0.4 N NaOH, collected, neutralized with HCl and counted in a liquid scintillation counter (Packard 1600 TR) with quench correction in Du Pont Aquasure scintillation cocktail.

^{3}H-aminoacid incorporation was assessed in a similar way by incubating the cultures for 16 hours in the presence of 0.5 μCi per well of ^{3}H-aminoacid mixture.

RNA extraction and analysis

RNA was extracted according to the guanidinium-thiocyanate method of Chomczinski and Sacchi[27]. Ten micrograms per lane of total RNA were run on an 0.8% agarose and 8% formaldehyde gel according to Maniatis, Sambrook et el[28]and transferred (Vacugene System, Pharmacia) onto a nylon membrane (Hybond N, Amersham International). The blot was hybridized with a random primer ^{32}P-dCTP- labeled probe (Amersham Multiprime Labeling Kit) in 0.5 mol/L sodium phosphate, 7% SDS at 60 °C. Autoradiography was performed on Kodak X-Omat S film with intensifying screens. The films were scanned with a Hewlett Packard IIC image scanner and quantified using a dedicated software program written by Dr Richard Prevost, Faculty of Pharmacy, University of Utrecht, The Netherlands. Scan values for t-PA and PAI-1 mRNA signals were normalized for the scan reading of the internal standard glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Ratios were expressed in arbitrary units.

DNA fragments used for hybridization

The PAI-1 probe was a 1.1 kB *Pst I* fragment of a human PAI-1 cDNA with a high homology to the rat PAI-1 gene[29] . The t-PA probe was a 2.0 kB *Bgl II* fragment from the human t-PA cDNA[30]. A 1.2 kB *Pst I* fragment from the rat GAPDH cDNA was used as an internal standard[31].

Statistical analysis

Data are expressed as the mean $\pm$ SD from the mean. Differences between experimental conditions were assessed by ANOVA and the Bonferroni t test. A value of $P \leq .05$ was considered significant.

RESULTS

PAI activity.

Ang II (300 nmol/L) stimulation induced a time-dependent increase in PAI activity in the supernatant of VSM SF cultures. A PAI activity increase compared to controls was detectable after 3 hours of Ang II treatment; PAI activity reached a plateau at 6 hours and remained constant for at least 24 hours (Fig. 1). The Ang II-induced increase in

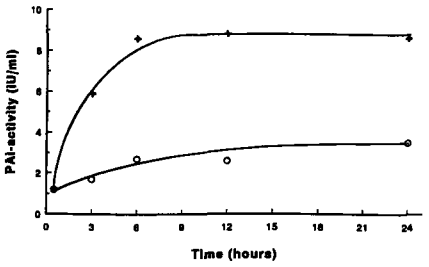


Figure 1.
Line plot: Time course of angiotensin II (300 nmol/L)-induced increase in plasminogen activator inhibitor (PAI) activity in rat aortic smooth muscle cell-conditioned medium (+) compared with control (o).
Typical experiment out of three.

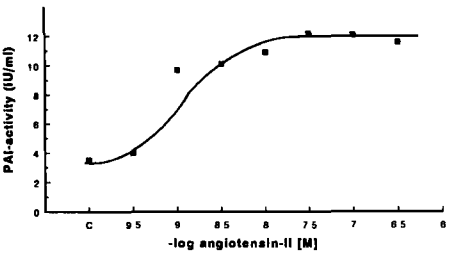


Figure 2. Dose-dependent increase in plasminogen activator inhibitor (PAI) activity in rat aorta smooth muscle cell-conditioned medium. Confluent cultures were kept serum-free for 48 hours before the addition of serum-free medium (C) or of angiotensin II at the specified concentration. Conditioned medium was collected after 16 hours. Typical experiment out of three.

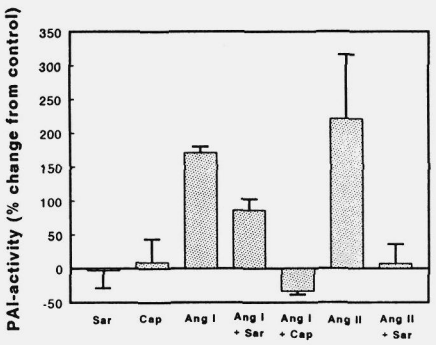
PAI activity was dose dependent, reaching a maximum at a concentration of 23 ± 11 nmol/L, n=3 and a 50% maximal effect at 3.3 ± 1.5 nmol/L, n=3 (Fig. 2). [Sar^1-Ile^8]angiotensin II, a specific competitive antagonist of Ang II, inhibited the maximal Ang II effect by 90 ± 9 %, n=3, P≤.001 (Fig. 3).

Recently, evidence has been accumulating that most of the components of the RAS can be synthesized by vascular cells. In particular, preliminary reports have described an ACE-like activity in VSM cultures (27). For this reason we compared the effects of Ang II and Ang I on PAI production in VSM cell cultures.[32] For this reason we compared the effects of Ang I and Ang II on PAI production in VSM cell cultures.

Ang I increased PAI activity in the supernatant of VSM cell-conditioned medium in a dose-dependent fashion (Fig. 4). The maximal effect of Ang I was comparable to the effect of a maximal dose of Ang II. To determine whether the Ang I effect was due to a direct effect or to the Ang II generated by the cleavage of Ang I, experiments were performed in the presence of [Sar^1-Ile^8]angiotensin II and of the ACE inhibitor captopril. [Sar^1-Ile^8]angiotensin II (300 nmol/L) inhibited the Ang I effect by 47 ± 10 %, (P≤.02, n=3). Captopril (5μg/mL) completely abolished the Ang I effect (Fig. 3).

Figure 3.

Bar graph showing the effect of angiotensin I (Ang I, 10 nmol/L) and II (Ang II, 10 nmol/L) on plasminogen activator inhibitor (PAI) activity in rat aorta smooth muscle cell-conditioned medium. The Ang II receptor antagonist [Sar^1-Ile^8]-angiotensin II (Sar, 1 μmol/L) significantly reduced both the Ang I and Ang II induced increase in PAI activity ($P \leq 0.02$, n=3 and $P \leq 0.001$, n=3, respectively). The inhibitor of angiotensin-converting enzyme captopril (Cap, 5μg/mL) completely abolished the Ang I effect. Data are expressed as percent change from control and represent the means of three independent experiments ± SD.



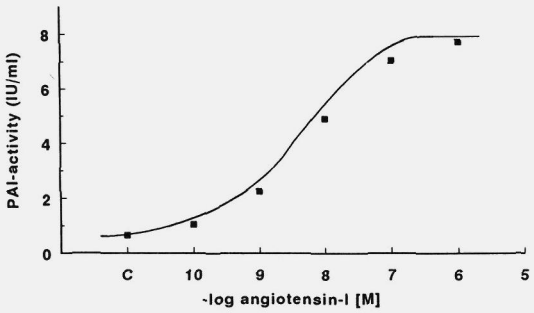


Figure 4.

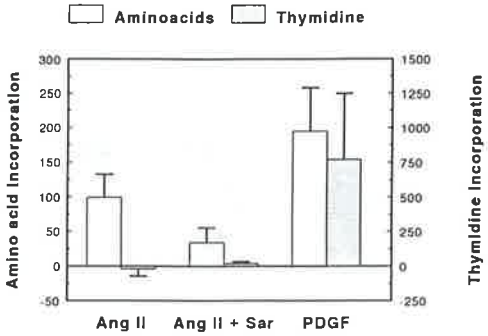
Line plot: Dose-dependent increase in plasminogen activator inhibitor (PAI) activity in rat aorta smooth muscle cell-conditioned medium. Confluent cultures were kept serum-free for 48 hours before the addition of angiotensin I at the specified concentration. Conditioned medium was collected after 16 hours. Typical experiment out of three.

[Sar^1-Ile^8]angiotensin II and captopril in the absence of Ang II caused a small non significant change in PAI activity from SF controls (-3.5 ± 25 % and 9.0 ± 34 % respectively).

^{3}H-thymidine and ^{3}H-aminoacid incorporation.

Ang II has been reported to cause hypertrophy and/or hyperplasia of VSM cells in vitro. In our system, under the described conditions, Ang II did not increase DNA synthesis as measured by ^{3}H-thymidine incorporation (a non-significant decrease of -20 ± 53 % , n=4, was observed). Ang II stimulation increased ^{3}H-aminoacid incorporation by 95 ± 38 % ($P \leq .02$ n=4) (Fig. 5). [Sar^1-Ile^8]angiotensin II (300 nmol/L) significantly reduced by 65 ± 23 % ($P \leq .05$, n=4) the Ang II induced ^{3}H-aminoacid incorporation. As positive control recombinant PDGF BB (1 nmol/L) was used. As expected, PDGF induced a several fold increase in ^{3}H-thymidine and ^{3}H-aminoacid incorporation (775±480%, $P \leq 0.01$, n=4 and 195±32%, $P \leq 0.01$, n=4, respectively).

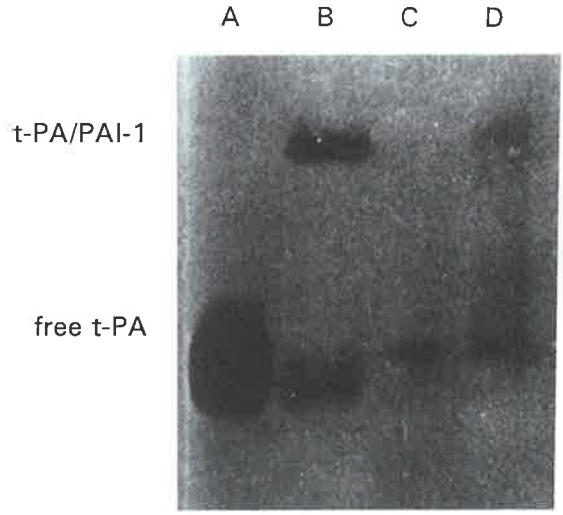
Figure 5.
Increase in ^{3}H-aminoacid incorporation, but not ^{3}H-thymidine incorporation after addition of angiotensin II to cultured rat aortic smooth muscle cells. Platelet derived growth factor increases both ^{3}H-aminoacid incorporation, and ^{3}H-thymidine incorporation and was added as a positive control. Data are expressed as total incorporated DPM (mean ± SD, n=3) after 16 hours (aminoacids) respectively 4 hours (thymidine).



Fibrin overlay zymography.

This method allows the detection of free plasminogen activators. However, even plasminogen activators in complex with PAI-1 can be detected by this method when they are present in relatively large amounts. This is most likely the result of in situ dissociation of the plasminogen activator from the inhibitor in the fibrin gel. In control conditions only t-PA was detected, while after Ang II treatment an area of lysis was seen in the region of the t-PA/PAI-1 complex (Fig. 6).

Figure 6.
Fibrin overlay zymography. Lane A: Human recombinant tissue-type plasminogen activator (t-PA, 20 mIU). Lane B: 50 μl of human endothelial cell-conditioned medium containing t-PA/PAI-1 complex and free t-PA. Lane C: 50 μl of rat vascular smooth muscle cell-conditioned serum-free medium. An area of lysis is observed in the region of free t-PA. Lane D: 50 μl of rat vascular smooth muscle cell-conditioned serum-free medium after treatment with 300 nmol/L angiotensin II for 16 hours. An area of lysis is observed in the regions of free t-PA and the t-PA/PAI-1 complex.



PAI-1 and t-PA gene expression.

Ang II stimulation of VSM cell cultures increased PAI-1 and t-PA mRNA compared with controls as assessed by Northern analysis. The mRNA increase was time-dependent with a similar time course for both PAI-1 and t-PA. The maximum was reached at 3 hours and the signal remained higher than controls for at least 18 hours (Fig. 7). The Ang II-induced PAI-1 mRNA increase was dose dependent, with a maximum effect at 100 nmol/L (Fig. 8A). This dose-response curve closely followed the PAI activity dose-response curve. The Ang II-induced t-PA gene expression was also dose dependent, displaying a dose-response relationship similar to that of PAI-1 mRNA. Since Northern blotting is a semi-quantitative technique, comparisons between different experimental situations are heavily dependent on the amount of total RNA loaded in each lane. This is particularly important in a dose-response experiment, where the induction of the signal at the intermediate and low doses of the agonist is relatively weak. For this reason we normalized the scan readings of the t-PA and PAI-1 mRNA signals for the scan readings of the internal standard GAPDH. Data are shown as the ratios between the t-PA or PAI-1 signals divided by the GAPDH signal and expressed in arbitrary units (Fig. 8B).

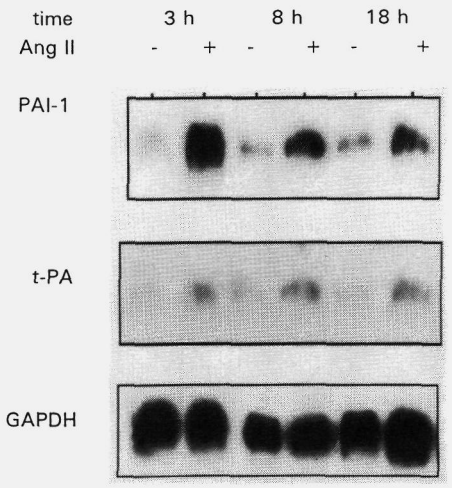
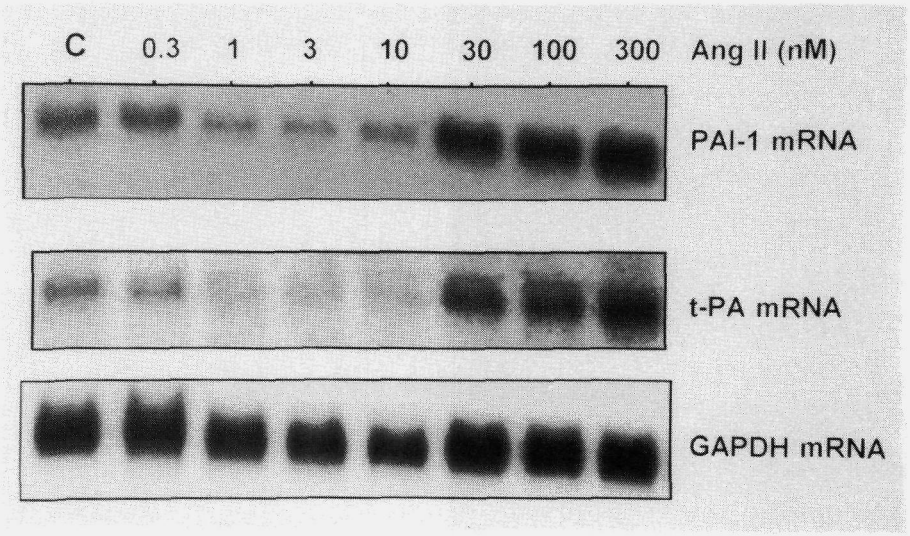


Figure 7.
Northern blot analysis showing the time course of angiotensis II (Ang II)-induced increase in plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA) mRNA. Vascular smooth muscle cell cultures were incubated for the specified time in control conditions (-) or in the presence of 300 nmol/L Ang II (+). Ten micrograms of total RNA were loaded in each lane. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) signal is shown as an internal standard.

To ascertain whether the observed effects of Ang II on PAI-1 and t-PA mRNA were direct or required the synthesis of an intermediate mediator, experiments were performed in the presence of the protein synthesis inhibitor cycloheximide. The angiotensin II-induced increase in PAI-1 and t-PA mRNA was unaffected by this agent, demonstrating that the Ang II effect is direct (Fig. 9).

DISCUSSION

These data show that the RAS can powerfully regulate the fibrinolytic balance in VSM cell cultures. PAI-1 and t-PA mRNA were increased by Ang II stimulation in a dose-dependent fashion, and [Sar^1-Ile^8]angiotensin II, a competitive Ang II antagonist, was capable of blocking 90 % of the Ang II-induced increase in PAI activity in the cell-conditioned supernatant. These data suggest that the Ang II effect is specific, ie, acting through a specific receptor(s). Future experiments using new selective nonpeptidic



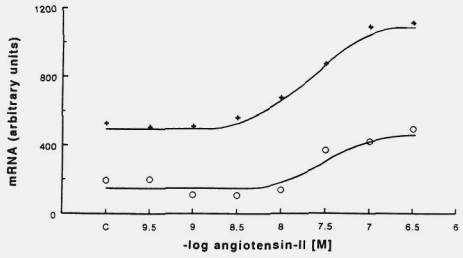


Figure 8.

A: Northern blot analysis of dose-related increase in plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA) mRNA induced by angiotensin II (Ang II). Vascular smooth muscle cell cultures were incubated for 6 hours in the presence of the specified concentrations of Ang II. Ten micrograms of total RNA was loaded in each lane. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) signal is shown as an internal standard. Lane 1, Control (C). Lane 2 through 8, Ang II at the specified concentrations.

B, Graph showing the dose-related increase in PAI-1 (+) and t-PA (o) mRNA induced by Ang II. The Northern blot films shown in panel A were scanned and the values obtained were normalized for the GAPDH reading. Data are expressed in arbitrary units.

receptor antagonists[33] should lead to the identification of the Ang II receptor subtype(s) involved. The concentrations used in our in vitro system are higher than the physiological concentrations reported in the literature. Recent determinations with both a radioimmunoassay and high-performance liquid chromatography are 14.1 ± 1 pmol/L in rat plasma and 6.6 ± 0.5 and 5.2 ± 2.8 (range, 0.4 to 52.2) pmol/L in human plasma.[34,35] However, very little is known about the local concentration of the RAS components within the vascular wall. Because it is likely that a local RAS is operating in the arterial wall, it is perfectly reasonable to hypothesize much higher concentrations of Ang II at this level. Our dose-response relation for Ang-II induced PAI activity production correlates well with the reported k_d for Ang II binding in a similar tissue culture system.[23] It has been shown that Ang II can induce the synthesis of vasoactive substances in VSM cells, thereby initiating an autocrine loop.[2,36] In particular, VSM cells in culture can produce PDGF-like molecules and PDGF synthesis and release are increased by Ang II.[36] We and others have previously shown that PDGF can elicit a powerful induction of PAI-1 mRNA and activity in cultured rat aortic VSM cells.[14,18] It could then be hypothesized that the Ang II effect we have observed on PAI-1 and t-PA production is indirect, requiring the previous synthesis of PDGF or another protein. The experimental evidence, however, suggests that this is not the case. First, the time courses of the PDGF[14] and Ang II effects on PAI-1 mRNA (Fig. 7) are similar, both peaking at around 3 hours. If the Ang II-induced effect required the previous synthesis of another protein it would be expected to take substantially longer. Secondly, the Ang II effect on PAI-1 mRNA was still observed in the presence of the protein synthesis inhibitor cycloheximide (Fig. 9).

The Ang II-induced increase in PAI-1 mRNA correlates well with the increase in PAI activity in the cell-conditioned medium. In the fibrin zymography an area of lysis, presumably due to dissociation of t-PA, was detected in the region of the t-PA/PAI-1 complex after Ang II stimulation. Ang II also induced a dose-dependent increase in the t-PA mRNA. t-PA activity, however, was not detected in measurable amounts in the cell-conditioned medium. This is not entirely surprising in an environment such as the cell-conditioned medium where t-PA can be readily inactivated by the large amount of PAI-1 present. Fibrin overlay zymography is a more sensitive semi-quantitative method to assess plasminogen activator activity. In basal conditions, small amounts of t-PA were detected with this method in the cell-conditioned medium. After Ang II stimulation, when PAI activity was markedly increased, t-PA appeared also in complex with PAI. Taken together, these data demonstrate that in the soluble phase the net Ang II effect was an increase in PAI activity. However, the Ang II effect on t-PA production could play an important role at a local level. Cell migration requires extracellular matrix digestion and plasminogen activation is an important cellular mechanism in the digestion of extracellular matrix proteins[37,38]. We have demonstrated that rat cultured VSM cells can digest extracellular matrices,

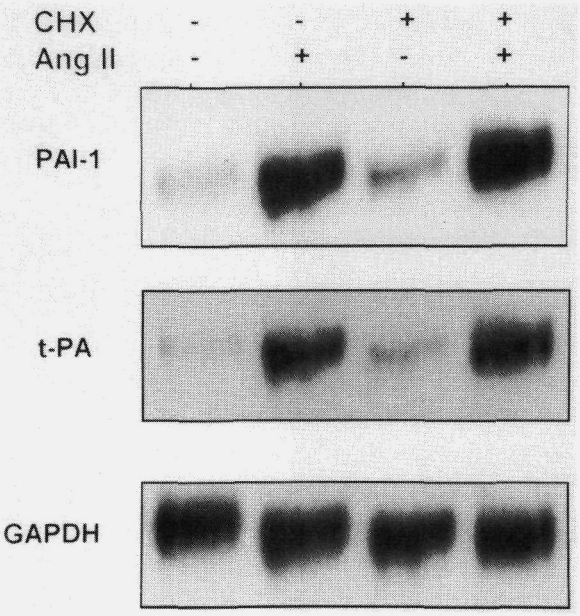


Figure 9.
Northern blot analysis demonstrating the lack of effect of the protein synthesis inhibitor cycloheximide (CHX, 10 μg/mL) on angiotensin II-induced (Ang II, 0.1 μmol/L) increase in plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA) mRNA. Vascular smooth muscle cell cultures were incubated for 3 hours in the absence (-) or in the presence (+) of the specified agents. Ten micrograms of total RNA was loaded in each lane. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) signal is shown as an internal standard.

with a mechanism which is at least in part plasminogen dependent[24]. Plasminogen activation was possible even in the presence of excess PAI activity, but required direct contact of the cells with the extracellular matrix substrate[24]. Cell surface receptor molecules have been demonstrated for plasminogen and for the two natural plasminogen activators, urokinase-like plasminogen activator (u-PA) and t-PA[39-42]. This cell-bound fibrinolytic system would provide both localization and, in the case of the t-PA receptor, protection of the cell-associated proteolysis from inhibition by PAI-1[41-43]. In vivo, t-PA is expressed by VSM cells during migration after balloon denudation in rat carotid arteries[44] and ACE inhibition can prevent intimal hyperplasia in rat carotid arteries after balloon denudation[45]. In vitro, Bell and Madri[46] have shown an increase in cell motility and in cell-associated plasminogen activator activity after stimulation with Ang II in cultured aortic bovine smooth muscle cells. These observations raise the possibility that the RAS could increase cell surface-associated proteolysis, while at the same time inhibiting plasminogen-dependent proteolysis in the surrounding milieu.
Clinical data have suggested an association between the RAS and the occurrence of myocardial infarction. Hypertensive patients with high plasma renin activity, and presumably high Ang II levels, have a higher risk of myocardial infarction compared to hypertensive patients with a low renin activity[9]. The DD genotype of the ACE is associated with increased plasma levels of Ang II[47] and represents a potent risk factor for myocardial infarction[10]. Similarly, raised plasma PAI activity has been shown to be more prevalent in young survivors of myocardial infarction[48], and to represent a risk factor for re-infarction in this patient population[49]. Recently, it has been reported that

Ang II infusion increases the circulating levels of PAI-1 antigen in normal volunteers without appreciable changes in the circulating t-PA antigen levels[22]. Preliminary data from the same group report an increase in the production of PAI-1 by cultured endothelial cells after stimulation with Ang II and the authors hypothesize that the PAI-1 increase seen in vivo could be of endothelial origin[50]. These data, however, are not necessarily in contrast with our in vitro data. Ang II could well have a similar effect on both endothelial and VSM cells. VSM cells represent the most abundant cell type in the vessel wall and their contribution to the systemic fibrinolytic balance could be important.

Recently, our understanding of the RAS has greatly expanded. The improvements in cellular and molecular biology approaches have shown the existence of several local renein-angiotensin systems. The vessel wall has been shown to be equipped with all the enzymes necessary to synthesize Ang II[1]. In particular, both renin[51] and ACE activities[32] have been described in cultured VSM cells and these cells have also been shown by in situ hybridization to express the mRNA for angiotensinogen[52]. We have confirmed that cultured rat VSM express ACE activity, as Ang I was as potent as Ang II in eliciting a PAI-1 response whereas this could be blocked by ACE inhibitors. Interestingly, both the ACE activity and the angiotensinogen mRNA expression have been shown to be increased by endothelial balloon denudation or by hormonal stimuli, which could be expected to be released after vessel wall injury[32,52].

Conclusions

A "vascular" RAS may profoundly influence medial VSM cells, and the novel finding that these cells produce PAI-1 and t-PA in response to Ang I and II has several potential implications. First, the modulation of plasminogen activation in the medial layer of arterial vessels is likely to affect the process of cell migration through it. This could influence the infiltration of inflammatory and tumor cells and also the migration of the VSM cells from the media to the intima during atherogenesis.

Second, the lysis of a vessel wall thrombus depends on the amount of plasminogen activators and inhibitors incorporated in the thrombus. The fibrinolytic factors produced at the interface between the clot and the vessel wall could therefore significantly affect the natural or exogenous lysis of arterial thrombi. It can be speculated that if confirmed in vivo, the effect of ACE inhibitors on PAI-1 production by smooth muscle cells could account at least in part for the reduction in cardiovascular events observed in certain patients subsets treated with these agents.

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CHAPTER 6.

CULTURED RAT AORTIC VASCULAR SMOOTH MUSCLE CELLS DIGEST NATURALLY PRODUCED EXTRACELLULAR MATRIX: INVOLVEMENT OF PLASMINOGEN-DEPENDENT AND PLASMINOGEN-INDEPENDENT PATHWAYS.

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ABSTRACT

Vascular smooth muscle (VSM) cell migration and proliferation play a major role in the development of atherosclerotic lesions, graft occlusion, and restenosis after angioplasty. Cell migration implies the digestion of the surrounding extracellular matrix. Cell-associated proteolysis has been extensively studied in neoplastic and inflammatory cells, but very little is known about the proteolytic properties of VSM. We have evaluated the ability of rat cultured VSM cells to solubilize [^{3}H]amino acid-labelled extracellular matrices produced by bovine VSM. When plated at a density of 30,000 cells/well in 24 multiwell plates, VSM cells were able to solubilize 63.3±7.0 % of the extracellular matrix after 10 days in culture. Extracellular matrix digestion occurred also when the cells were cultured in plasminogen-depleted serum, but was higher in the presence of 10 μg/ml of purified plasminogen (net percent digestion, after the subtraction of the appropriate control: 8.6±3.0 vs 21.2±3.5 after 3 days in culture, $p \leq 0.005$, respectively). The involvement of other enzymes in addition to plasmin is confirmed by the ability of VSM cells to degrade extracellular matrices from which the plasmin-sensitive component was removed with plasmin pretreatment. Rat VSM cells were able to solubilize 52.3±2.0 % of this residual extracellular matrix-associated radioactivity after 6 days in culture vs 26.1±1.5 in the control dishes ($p \leq 0.01$, $n=5$). Cell contact was required for extracellular matrix degradation: cell-conditioned medium did not have any effect on extracellular matrix digestion. Similarly, no extracellular matrix digestion was observed when the cells were cultured on porous membranes suspended at 1mm above the extracellular matrices. These experiments demonstrate that VSM cells can potently digest naturally produced extracellular matrices and that both plasminogen-dependent and plasminogen-independent mechanisms requiring cell contact are involved. A better knowledge of the fibrinolytic/ proteolytic properties of VSM cells might contribute to the design of new strategies to prevent VSM cell migration from the intima to the media in the vascular wall.

Key words: Extracellular matrix degradation, vascular smooth muscle, plasminogen, proteolysis.

INTRODUCTION

Vascular smooth muscle (VSM) represents the most abundant cell type in the arterial wall. Recently our ideas about the role played by smooth muscle cells have greatly changed because these cells have been shown to produce several biologically active substances including mitogens,[1-3] cytokines,[4-6] and factors of the fibrinolytic cascade,[7,8] VSM cell migration into and proliferation in the arterial intima are believed to play a major role in restenosis after angioplasty, graft occlusion and atheromatous plaque formation.[9-12] Cells that are able to migrate must be able to digest the extracellular matrix proteins within which they are embedded. It is known that neoplastic[13-15] and inflammatory cells such as macrophages and polymorphonucleates[16-18] are able to digest extracellular matrices, and these cells have been studied in several in vitro models. Despite the fact that VSM cells are able to migrate and proliferate in the arterial wall, little is known about their proteolytic properties. This is an important lack of information because inhibition of VSM cell-associated proteolysis might represent a potential way of inhibiting the migration of these cells into the intima that eventually leads to obstruction.

Degradation of matrix components is believed to be the result of a proteolytic cascade which involves the cooperation of serine protease, cysteine protease, and metalloproteinases,[14, 19] and modulation by their inhibitors.[20] Plasmin is a rather nonspecific serine protease which is very efficient in cleaving not only fibrin, but also several extracellular matrix glycoproteins.[16] It can also activate procollagenase[21] and cooperate with elastase in the digestion of elastin.[22] It is now established that plasminogen activation plays a central role in the extracellular matrix digestion by neoplastic and inflammatory cells,[14, 23] and its inhibition can prevent cell migration and metastasis in some in vitro models.[19, 24]

Recently we have been able to demonstrate that VSM cells are able to synthesize in vitro and in vivo the enzymes necessary to modulate plasminogen activation.[8] They express the mRNA for tissue-type plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1). PAI-1 was present in the cell-conditioned medium both in an active free form and in complex with t-PA. In this paper we demonstrate that VSM cells are able to digest naturally produced extracellular matrices, solubilizing both plasmin-sensitive and plasmin-insensitive extracellular matrix proteins.

Materials and Methods

Cell culture

Rat aortic VSM cells were obtained by enzymatic dissociation according to the method of Gunther and Gimbrone.[25] These cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% neonatal calf serum. Passages 6-20 were used for the experiments.

Bovine smooth muscle cells were a kind gift of David Crossman, Clinical Research Centre, Harrow, Middlesex, U.K. They were obtained from calf aorta with a tissue explant technique[26] and cultured in the same medium supplemented with 10 % fetal calf serum. Passages 10 to 25 were used.

M14[27] and BLM[28] are two highly metastatic cell lines obtained from human melanoma metastasis. They were kindly provided by Dr G.N.P. van Muijen, Department of Pathology, University Hospital, Nijmegen, The Netherlands. Both cell lines have been characterized for extracellular matrix digestion.[29] They were cultured in DMEM with 10% fetal calf serum, in an essentially identical way to the VSM cells.

Plasminogen depletion

Human serum was plasminogen depleted by lysine-Sepharose affinity chromatography following the method of Deutsch and Mertz.[30] Aliquots of 40 ml of human serum were three times passed over a C 16/20 Pharmacia column containing 5 g of lysine-Sepharose (bed volume 8.3 ml) at a rate of 30 ml/h. Lysine-bound plasminogen was displaced after each elution with 0.2 M ϵ-amino caproic acid. The eluate was continuously monitored in a UV spectrophotometer at a wavelenght of 280 nm and the plasminogen peak was no longer detectable after the second elution. The effectiveness of the procedure was confirmed by assessing plasminogen activity in the final eluate with a streptokinase activation chromogenic assay kit (Kabi, Uppsala, Sweden).[31]

Production of labelled extracellular matrices

Labelled extracellular matrices were prepared according to the methods of Jones et al[32] and Chapman et al.[16] Briefly, bovine smooth muscle cells were plated in 24 multiwell cluster plates and grown for 7-9 days until they reached confluence. The cultures were then grown for further 4 days in DMEM containing 10% fetal calf serum supplemented with 1 μCi/ml [^{3}H]amino acids (Amersham) with the medium changed every other day. The cells were then lysed with 0.5% Triton X-100 in phosphate buffer pH 7.4 for about 10 min. The cytoskeletal elements and the nuclei were removed with a 5 min incubation in 25 mM NH_4OH. These steps were carefully monitored by phase-contrast microscopy. The cultures were washed twice with sterile distilled water, rinsed with 75% ethanol-25% water, dried at room temperature for 8-10 minutes and stored at -20°C. Before being used for the experiments, the [^{3}H]extracellular matrix-coated dishes were

incubated in serum-free medium for 6 hours and then washed twice.

Composition of extracellular matrix.

The composition of the extracellular matrix was estimated by enzymatic digestion as described by Jones et al.[32] Digestion experiments were performed with the following enzymes: trypsin (type III, Sigma Chemical Co., St.Louis, Mo.), 20 μg/ml; elastase (type I from porcine pancreas, Sigma), 5 μg/ml; Collagenase (type I *from Clostridium difficilis* Sigma), 10 μg/ml; and plasmin (10 WHO units/μg protein, Sigma), 10 μg/ml. Elastase and collagenase were used in the presence of 10 μg/ml of soybean trypsin inhibitor (Sigma). All enzymes were dissolved in Hank's balanced salt solution. Preliminary experiments determined that treatment for 2h at 37°C with the specified enzyme concentrations was sufficient to completely remove the enzyme-sensitive components from the extracellular matrices.

Thymidine incorporation and plating efficiency.

Rat aortic VSM cells were plated at a density of 10,000 cells/dish on extracellular matrix-coated 24 multiwell dishes in the presence or in the absence of 10 μg/ml of plasminogen. After 24 h, 1 μCi of [^{3}H]thymidine was added to the dishes. After further 24 h, the dishes were washed twice with 0.5 ml of 0.9 % saline, once with 0.5 ml of 10 % trichloroacetic acid, and then again twice with 0.9 % saline. The cells were dissolved in 0.4 N NaOH, acidified with the proper amount of 1 N HCl and the radioactivity counted in a Beckman 1803 LS liquid scintillation counter.

Cells pre-labelled with [^{3}H]thymidine were plated on dishes coated with non-radioactive extracellular matrix at a density of 20,000 cells/ well. After 24 h the cultures were washed, dissolved and counted as for thymidine incorporation.

Extracellular matrix digestion

Extracellular matrix digestion experiments were performed by plating the specified number of cells on dishes coated with radiolabelled extracellular matrices. Control wells were treated identically to the experimental ones, but no cells were plated. The medium was changed at the specified intervals and the radioactivity that had been released into the supernatant was counted. At the end of the experiments, the extracellular matrix remaining in each well was dissolved with 0.4 N NaOH, acidified with the appropriate amount of 1N HCl and counted. Extracellular matrix digestion was expressed as a percentage of the total amount of radioactivity initially present in each dish. The latter was defined as the radioactivity recovered in the supernatant plus the amount of radioactivity recovered in the dish at the end of the experiment.

Plasminogen reconstitution experiments were performed by adding 10 μg of purified plasminogen (P5661, purified by lysine-Sepharose affinity chromatography from human plasma and containing less than 0.0001 unit plasmin per unit plasminogen, Sigma) to

extracellular matrices prepared from bovine smooth muscle cultures grown in DMEM supplemented with plasminogen-depleted human serum.

The ability of VSM cells to digest extracellular matrix components that are sensitive to proteinases other than plasmin was assessed by plating VSM cells on matrices from which the plasmin-sensitive component had been removed with plasmin digestion. These experiments were performed both in plasminogen-free conditions and in the presence of 10 μg/ml purified plasminogen. Plasmin contamination of plasmin-pretreated extracellular matrices was assessed by monitoring the cleavage of the chromogenic peptide S2251 (Kabi) at a wavelenght of 405 nm. An aliquot of S2251 to a final concentration of 0.15 mg/ml was added directly to the extracellular matrix-coated dishes, which were treated and washed as previously described but in the absence of cells. The sensitivity of the method was assessed by adding a known amount of plasmin to extracellular matrix-coated dishes. An amount of plasmin $\geq$100 pg/ml was reliably detectable with this method.

A crude cell homogenate was prepared by scraping the cells from confluent 35 mm dishes, resuspending them in 0.5 ml of 10 % neonatal calf serum medium and homogenizing them in a glass homogenizer.

The relevance of cell contact in extracellular matrix digestion by VSM was evaluated by growing VSM cells on porous membranes (Transwell, pore diameter 3 μm, Costar Co., Cambridge, Mass) suspended at 1 mm above the labeled extracellular matrices. The radioactivity released in the supernatant was counted as previously described. Medium conditioned for 24 h by rat VSM cell cultures was also used on extracellular matrix digestion experiments.

Statistical analysis

Data are expressed as the mean $\pm$SEM

Student's *t* test for paired data was used for statistical analysis.

RESULTS

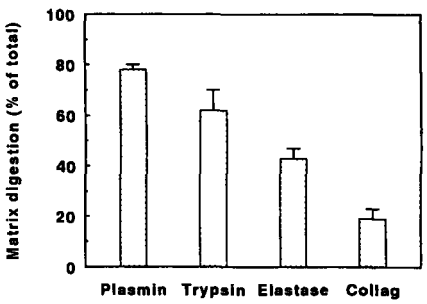
Composition of extracellular matrix.

Trypsin, elastase and collagenase respectively solubilized 62.0$\pm$8.1%, 43.0$\pm$4.4%, and 21.0$\pm$3.7% (mean$\pm$SEM, n=4) of the total extracellular matrix-associated radioactivity. The amount of extracellular matrix-associated radioactivity sensitive to plasmin was 74.4$\pm$1.0 % of the total (mean$\pm$SEM, n=4, Figure 1).

Extracellular matrix digestion

Rat aortic VSM cells can digest naturally produced extracellular matrix (Figure 2). When plated at a density of 30,000 cells per well, the cultures became confluent after about 3-4 days. As characteristic of smooth muscle cells, these cultures continued growing to form a "hill and valley" pattern.

When 30,000 rat aortic VSM cells were plated on dishes coated with labelled matrix, theamount of radioactivity released in in the supernatant was 64.3 ± 5.8 % (mean±SEM, n=5) of total after 10 days in culture. Some extracellular matrix-associated radioactivity was nonspecifically released in the supernatant of the control dishes where only medium was added: 27.4±6.7% of total after 10 days in culture (mean±SEM, n=5). However the amount of extracellular matrix digestion in the presence of cells was significantly higher than the control value at all time points measured ($p \leq 0.01$, n=5, Figure 2). The digestion of extracellular matrices by VSM



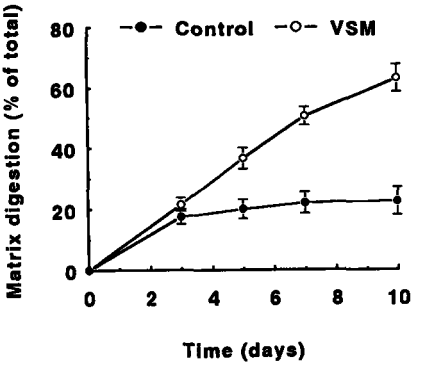


Figure 1 (left). Bar graph showing the composition of extracellular matrices as determined by enzymatic digestion. [^{3}H]Amino acid-labeled extracellular matrices produced by bovine aortic vascular smooth muscle cells were treated with one of the following enzymes for 2 hours at 37°C: plasmin (10 WHO units/mg protein), 10 μg/ml; trypsin (type III), 20 μg/ml; elastase (type I from porcine pancreas), 5 μg/ml; collagenase (type I from Clostridium difficilis), 10 μg/ml. Total radioactivity was determined for each experiment as the released amount plus the amount recovered by dissolving the extra-cellular matrices with 0.4N NaOH at the end of the enzymatic digestions. Data are the mean±SEM of triplicates determined in 4 separate experiments.

Figure 2 (right). Graph showing cumulative digestion of extracellular matrices by vascular smooth muscle (VSM) cells. Cells (30,000 per well) were plated on 24 multiwell dishes coated with labelled extra-cellular matrices. The cells were grown in DME medium containing 10% (v/v) neonatal calf serum. In the control dishes, only the medium was added. The medium was changed at various intervals and the amount of radioactivity present in the supernatant counted. The total radioactivity of each well was determined by adding the radioactivity recovered in the medium to the radioactivity recovered by dissolving the extracellular matrices with 0.4 N NaOH at the end of the experiment. The data represent triplicate determinations (mean±SEM) in five separate experiments. The extracellular matrix digestion in the presence of cells was significantly higher than in the control wells at all time-points ($p \leq 0.01$, n=5).

cells was also compared with that by two human melanoma cell lines (BLM and M14), selected for their metastatic and proteolytic properties.[27, 28, 29] In parallel experiments, after 5 days in culture, rat aortic VSM cells had digested 54.0±0.5 % (mean±SEM,

n=3) of the total extracellular matrix-associated radioactivity. BLM and M14 cells had digested 86.0±1.0% and 82.3±1.0%, respectively (mean±SEM, n=3). A typical extracellular matrix digestion time course experiment for all three cell lines is shown in Figure 3.

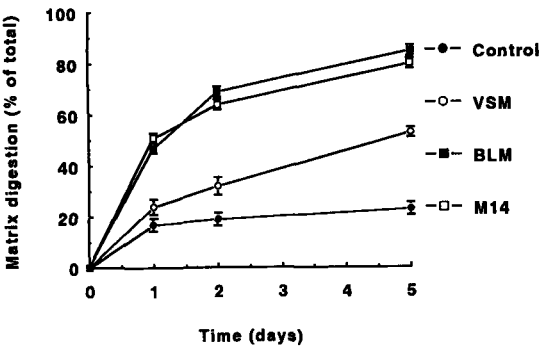


Figure 3. Graph showing extracellular matrix digestion by vascular smooth muscle (VSM) cells: comparison with human malignant melanoma cell lines (BLM and M14). All three cell types were plated at an initial density of 60,000 cells per well in in Dulbecco's modified Eagle medium containing 10% neonatal calf serum. In the control dishes, only medium was added. The amount of extracellular matrix digestion was determined as described in Figure 2. Values are mean±SEM of triplicate determinations of a typical experiment out of a total of three.

In our experimental system cell contact is an absolute requirement for extracellular matrix digestion. This is proved by the demonstration that: 1) the medium conditioned by confluent rat aortic VSM cells did not increase the release of extracellular matrix-associated radioactivity into the supernatant (Figure 4) and 2) no difference in the release of radioactivity from the extracellular matrix was observed between the control wells and the Transwells containing the porous membranes with the VSM cultures (Figure 4). To exclude the possibility that cell death with the release of neutral lysosomal proteinases could account for some extracellular matrix digestion, experiments were performed using a crude cell homogenate. A small increase in the amount of the release of extracellular matrix-associated counts was observed, but it was not statistically significant (Figure 4).

Plasminogen dependency of extracellular matrix digestion.

To assess the role of plasminogen activation in extracellular matrix digestion by VSM cells, experiments were performed in the presence of plasminogen-depleted serum. To avoid the possible presence of plasminogen bound to extracellular matrix proteins,[32] the extracellular matrices used in these experiments were produced by bovine smooth muscle cell cultures grown in the plasminogen-depleted serum. In these experiments cells were plated at 60,000 cells per well and the digestion was monitored daily for 3 days. These experiments demonstrate that some extracellular matrix digestion can occur in plasminogen-free conditions (Figure 5A). The amount of extracellular matrix-

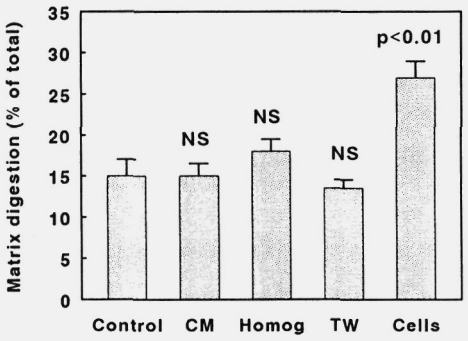


Figure 4. Bar graph demonstrating the absolute requirement of cell contact for extracellular matrix digestion by vascular smooth muscle cells. Control, medium containing 10% neonatal calf serum; CM, medium conditioned for 48h by vascular smooth muscle cultures; Homog, crude smooth muscle cell homogenate prepared by scraping cultures and homogenizing the cells in a glass homogenizer; TW, vascular smooth muscle cells growing on porous membranes 1 mm above the extracellular matrices; Cells, vascular smooth muscle cells plated at an initial density of 60,000 cells per well. The amount of extracellular matrix digestion was determined as in Figure 2. The homogenate of 1,000,000 cells was diluted in 1 ml serum-free medium. The appropriate medium was changed daily for 3 days. Values (mean±SEM, n=4) correspond to the cumulative amount of extracellular matrix digestion observed at day 3 and determined as in figure 2.

associated radioactivity released in the supernatant (percentage of total) is significantly higher than the control value at day 2 and 3: day 2, 23.3±3.5% versus 16.2±2.0%; respectively; day 3, 27.3±3.2% versus 18.7±2.4%, respectively (mean±SEM, $p \leq 0.01$ for both, n=5).

Reconstitution experiments were also performed by adding a known amount of purified plasminogen to plasminogen-depleted cultures. Purified plasminogen preparations, however, can contain small amounts of plasmin which can release radioactivity from the extracellular matrices. Alternatively, plasminogen could be activated to plasmin by some plasminogen activator present in the extracellular matrix. In fact, the amount of radioactivity released from the extracellular matrices in the absence of cells was higher in the control wells in which plasminogen was added compared with the wells without plasminogen: day 1, 18.5±2.4% versus 11.5±1.2%, respectively, $p \leq 0.01$, respectively; day 2, 23.4±3.2% versus 16.2±2.0%, respectively, $p \leq 0.01$; day 3, 26.1±3.5% versus 18.7±2.4%, respectively, $p \leq 0.005$ (mean±SEM, n=5). To take this into account, data were expressed as the net cell-associated extracellular matrix digestion (percentage of total) defined as the amount of radioactivity released in the presence of cells with or without plasminogen minus the amount of radioactivity present in the appropriate control condition, i.e. with or without plasminogen (Figure 5B).

The addition of 10 μg/ml purified plasminogen to the cultures significantly increased the net cell-associated extracellular matrix digestion (percentage of total) at all time-points (Figure 5B). Day 1, 11.7±2.8% versus 2.7±0.9%, respectively, $p \leq 0.03$; day 2, 15.4±3.1% versus 5.5±1.1%, respectively, $p \leq 0.02$; day 3, 21.2±3.5% versus 8.6±3.0%, respectively, $p \leq 0.005$, (mean ±SEM, n=5) These differences are not due to an effect of plasminogen on plating efficiency or the growth rate.

Plating efficiency was equally high both in the absence and in the presence of plasminogen: 95.3±0.4% and 93.1±1.6% respectively (mean±SEM, n=4, p=NS).

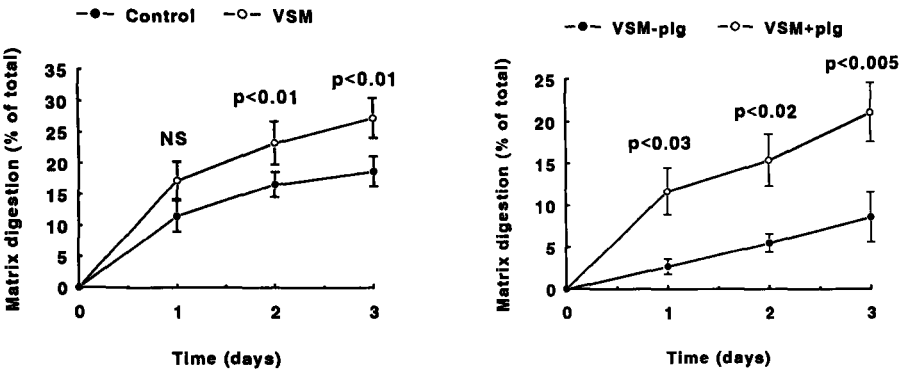


Figure 5. Panel A (left): Graph showing digestion of extracellular matrices by vascular smooth muscle cells (VSM) in the absence of plasminogen. Cells (60,000) were plated in Dulbecco's modified Eagle medium supplemented with 10% (v/v) human plasminogen-depleted serum. To exclude the presence of plasminogen bound to the extracellular matrix, these experiments were performed with extracellular matrix produced by cultures grown in medium supplemented with plasminogen-free human serum (mean±SEM, n=5).
Panel B (right): Graph comparing net extracellular matrix digestion by vascular smooth muscle cells in the absence and in the presence of plasminogen. Net extracellular matrix digestion was defined as the amount of radioactivity released in the presence of cells minus the amount of radioactivity present in the appropriate control condition, i.e. in the presence or in the absence of plasminogen. Plasminogen-free extracellular matrices prepared as described in panel A were used. The medium with or without plasminogen was changed daily for 3 days. The net amount of cell-associated extracellular matrix digestion is higher in the presence of plasminogen at all timepoints (mean±SEM, n=5)

Thymidine incorporation was also not affected by plasminogen. The amount of [^{3}H]thymidine incorporated in 24h in the absence and in the presence of plasminogen was 261±42 and 280±23 * 10^3 dpm per well, respectively (mean±SEM, n=4, p=NS).

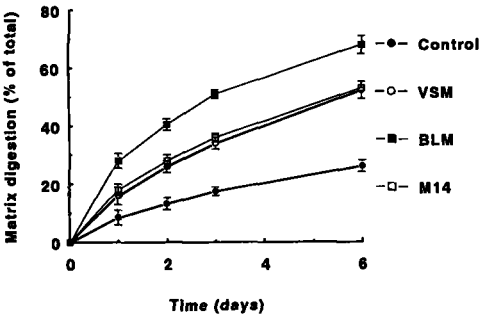


Figure 6. Graph showing the digestion of plasmin-insensitive extracellular matrix components by vascular smooth muscle (VSM) cells and human malignant melanoma cell lines (BLM and M14). The labelled extracellular matrices were subjected to a pretreatment with 10 μg/ml purified plasmin to completely remove any plasmin-sensitive component from the matrices. All three cell types were plated at an initial density of 60,000 cell per well and the extracellular matrix digestion was assessed as previously described in Figure 2. The residual plasmin-insensitive radioactivity was considered 100%. The extracellular matrix digestion in the presence of all 3 cell types was significantly higher than in the control wells at all time-points ($p \leq 0.01$, mean±SEM, n=5).

Digestion of plasmin-pretreated matrices

Rat aortic VSM cells were able to digest extracellular matrices from which the plasmin-sensitive components had been previously removed (Figure 6). In this series of experiments, 22.6±1.0% of the initially extracellular matrix-associated radioactivity was not removed by plasmin treatment. After 6 days in culture, rat aortic VSM cells were able to digest 52±33.6% of this residual extracellular matrix-associated radioactivity. The amount of extracellular matrix radioactivity non specifically released in the supernatant was 26.1±2.0 %. The difference between the extracellular matrix digestion in the VSM-containing dishes and the control dishes was highly significant ($p \leq 0.01$, n=5) at all time-points measured.

Both tumor cell lines tested were able to digest a significant amount of plasmin-insensitive extracellular matrix (Figure 6). After 6 days in culture the extracellular matrix digestion associated to the M14 cells was 53.0±1.2%, an amount very similar to that digested by the rat aortic VSM cells. The BLM cells were somewhat more potent, with a digestion of 67.9±3.1% at 6 days (Figure 6)

Plasmin can indirectly affect the digestion of substrates that are not directly sensitive to it, for instance by cleaving and activating a different protease. For this reason, the digestion of plasmin-pretreated extracellular matrix was assessed in the presence and in the absence of plasminogen. Plasmin contamination of plasmin-pretreated extracellular matrix was assessed and found to be as low as 160±16 pg per well (n=3). Serum completely inhibited this residual plasmin activity: when plasmin contamination was assessed in the regular medium used in the extracellular matrix digestion experiments and containing 10% fetal calf serum, no activity was seen. In the absence of plasminogen the digestion of plasmin-pretreated extracellular matrices was significantly higher in the presence of cells at all time-points measured: day 1, 36.0±1.4% versus 26.8±0.9, respectively, $p \leq 0.01$; day 2, 48.5±2.2% versus 34.3±1.3%, respectively, $p \leq 0.001$; day 3, 57.0±2.1% versus 39.0±3.6%, respectively, $p \leq 0.001$; day 6, 72.8±1.8% versus 48.0±2.4%, repectively, $p \leq 0.001$ (mean±SEM, n=4) (Figure 7). The addition of plasminogen to the cultures did not increase further the amount of extracellular matrix digestion (Figure 7).

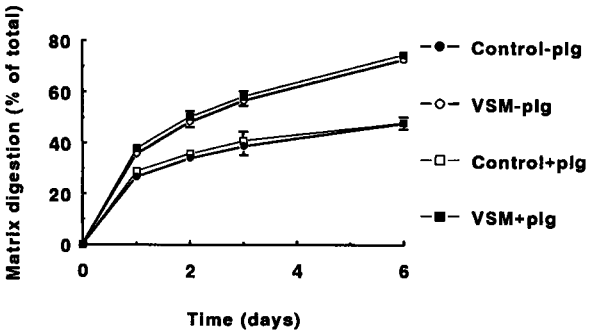


Figure 7. Graph showing the effect of plasminogen on the digestion of plasmin-insensitive components of extracellular matrix by vascular smooth muscle (VSM) cells. Digestion experiments of plasmin-pretreated extracellular matrices (as in Figure 6) were performed in the absence and in the presence of purified plasminogen as indicated. The appropriate controls (with or without plasminogen) are also shown. In the absence of plasminogen the extracellular matrix digestion was significantly ($p \leq 0.01$, $n=5$) higher in the presence of cells at all time points.The addition of plasminogen did not increase further the amount of extracellular matrix digestion.

DISCUSSION

This study demonstrates that cultured rat aortic VSM cells can potently digest both plasmin-sensitive and plasmin-insensitive components of naturally produced extracellular matrices.

A plasminogen-dependent component in extracellular matrix digestion by VSM cells is clearly demonstrated by the fact that the addition of plasminogen to plasminogen-depleted cultures significantly enhances extracellular matrix digestion. Several cell types are able to convert plasminogen to its active form plasmin by producing one or both of the two specific plasminogen activators, tissue-type plasminogen activator (t-PA)[33] and urokinase-like plasminogen activator (u-PA).[34] Both these activators can interact with their specific inhibitors, PAI-1 [35, 36] and plasminogen activator inhibitor type 2.[37, 38] Recently, we have shown that rat aortic VSM cells produce large amounts of PAI-1 both in vivo and in culture conditions identical to those of the extracellular matrix digestion experiments performed in this study.[8] High levels of plasminogen activator inhibitor activity were present in the cell-conditioned medium, but no t-PA activity was detected. t-PA/PAI-1 complexes were detected by immunoblotting and fibrin autography. In the present series of experiments, the demonstration that VSM cell-associated extracellular matrix degradation is at least in part plasminogen dependent also implies that these cells are able to promote plasminogen activation in an environment where the balance is overwhelmingly in favor of plasminogen activator inhibitor activity. Generally, plasminogen activation is not a reaction which occurs unchecked in the circulation; rather, it requires that all the components to be bound or "anchored" to a substratum or to a co-factor. In the fibrinolytic cascade the action of the inhibitors is partially escaped by the binding of plasmin to fibrin[39] and by the enhancement of t-

PA activity by its binding to fibrin.[40] Similarly, in cell-associated proteolysis, the enzymatic activity is localized at the interface between the cells and the substrates. In many cell types, cell contact is either necessary or at least substantially enhances the degradation of the extracellular matrix substrates.[12, 41, 42] In the case of VSM cells, contact is an absolute requirement to digest the extracellular matrix components: VSM cell-conditioned medium was not effective in digesting extracellular matrix and growing the VSM cells on porous membranes in proximity to (1mm), but not in direct contact with, the extracellular matrix was not sufficient to achieve any extracellular matrix digestion. Specific binding of plasmin(ogen) to a variety of cells has been demonstrated, including monocytes/macrophages and endothelial cells.[43-45] Receptor-bound plasminogen can be converted to plasmin by physiological PAs such as t-PA and u-PA.[45, 46] This mechanism would provide a means of restricting plasmin proteolysis to a localized environment. Alternatively, plasmin generated from plasminogen in the soluble phase can bind to the same receptor with the same affinity as its zymogen.[46] Recently a specific receptor to which u-PA binds with high affinity has been purified and characterized.[47] Receptor-bound u-PA is effective in activating plasminogen and in particular receptor-bound plasminogen.[48] For this reason it is believed to play a role in localizing proteolysis to the cell surface. Another function of the u-PA receptor has been postulated, namely the shielding of the u-PA molecule from PAI-1. According to recent evidence, however, it seems that this protection is at most partial.[49] Specific high-affinity binding of active t-PA to the cell surface has also been described in several cell types, including endothelial and smooth muscle cells.[50, 51] The putative receptor for t-PA has not yet been characterized to the same extent as the u-PA receptor, but it appears to confer a high degree of protection from PAI-1.[50] On the basis of our data it is not possible to establish by which mechanism VSM cells activate plasminogen in our system. Because digestion requires contact, the activation of plasminogen by a plasminogen activator bound to the cell surface seems likely. The demonstration by fibrin autography of t-PA synthesis by these cells, and the lack of evidence by this same technique for u-PA production, make t-PA a more likely candidate.

VSM cells can solubilize a significant amount of extracellular matrix also in plasminogen-free conditions. All precautions were taken to exclude the presence of plasminogen from the experimental system. As this enzyme binds to fibrin or extracellular matrix proteins,[52] the bovine VSM cell culture from which the labeled extracellular matrices were prepared were also grown in plasminogen-depleted serum. However, the presence of catalytic amounts of plasminogen in the plasminogen-depleted serum or in the extracellular matrix, or the production of small amounts of plasminogen by the rat VSM cells themselves cannot be excluded. For these reasons, extracellular matrices were predigested with purified plasmin to completely remove the plasmin-sensitive component, and subsequently seeded with rat VSM cells. Also, in these conditions a significant amount of extracellular matrix digestion was observed,

suggesting that proteases capable of digesting substrates other than those sensitive to plasmin are involved (Figure 6). Plasmin can indirectly be important in the proteolysis of substrates which are not directly sensitive to it. For instance, it can modulate collagen proteolysis by activating stromelysin and type IV procollagenase.[21] Plasmin can also cooperate with elastases in the digestion of elastin.[22, 41] For these reasons, the role of plasminogen activation in the digestion of plasmin-insensitive substrates was also investigated. A significant amount of extracellular matrix degradation took place also in plasminogen-free condition, supporting the existence of a truly plasmin-independent mechanism for extracellular matrix digestion in VSM cells (Figure 7). The extremely small amount of residual plasmin contamination in plasmin-predigested extracellular matrices is not likely to play a significant role because it is inhibited by the fetal calf serum present in the culture medium. It cannot be excluded, however, that cell surface-bound plasmin might have a different specificity, not being inhibitable by fetal calf serum, and therefore contribute to the extracellular matrix digestion in these experimental conditions. This hypothesis however seems unlikely because of the extremely small amount of plasmin residual in the system compared to the relatively high amount of extracellular matrix digestion observed in these conditions. When used in a cell-free system this amount of plasmin is not able to cause any extracellular matrix digestion (data not shown). This pathway also requires cell contact, and mechanisms similar to the plasmin-dependent pathway to localyze proteolysis can be hypothesized.

The amount of extracellular matrix digestion by one cell type in a particular experimental system is difficult to compare with other cell types in different experimental conditions. The release of radioactivity from labeled extracellular matrices is usually monitored over days rather than hours and during this time the number of cells is continuously changing. Most investigators have not normalized the amount of radioactivity released in the supernatant for the total amount of radioactivity present in the extracellular matrices. The use of naturally produced matrices versus purified substrates also complicate the comparison of data. For these reasons we compared the VSM cells with two highly metastatic human melanoma cell lines, which have been proven to be very potent in digesting extracellular matrix produced by bovine VSM cells.[29] Both neoplastic cell lines showed a large amount of extracellular matrix digestion (Figure3). VSM cells were also very potent and after 5 days in culture they digested an amount of matrix equal to about 50% of the amount digested by the BLM or M14 cells. Two factors have to be taken into account when comparing these two melanoma cell lines with non transformed cells. 1) Despite being plated at the same initial density, the growth rate of the transformed cells is likely to be much faster than that of the VSM cells. 2) The two cell lines tested were the most potent among a series of malignant melanoma cells evaluated for their ability to digest labeled matrices.[29] The study of the mechanisms underlying extracellular matrix digestion by human melanoma

cell lines are beyond the purposes of this paper. However it seems that all the cell lines tested can digest both plasmin-sensitive and plasmin-insensitive substrates (Figure 6). VSM cells are not the only nontransformed cell line able to digest extracellular matrices in culture. Macrophages[16] and endothelial cells[53] have been found to be able to digest both purified substrates such as elastin and naturally produced extracellular matrices. Conversely, not all cells seem to be able to digest extracellular matrix. For instance human skin fibroblast[54] and even tumor cells[29] have been shown not to digest extracellular matrix substrates in an appreciable amount in experimental conditions similar to those described in this study. It cannot be excluded that even these cells could be able to do so in vivo when cooperating with other cell types. Ours represents the first study to demonstrate this capability by VSM cells and to investigate the mechanisms by which this occurs. More studies are necessary to further elucidate the mechanism(s) of plasminogen activation and the enzymes involved in the digestion of the plasmin-insensitive substrate(s) and to investigate how extracellular matrix digestion can be inhibited. We believe that the interest of this study is in indicating a new potential way to prevent VSM cells migration. For example, this approach might be promising in the case of restenosis after angioplasty, where interference with physiological processes such as the healing of surgical wounds would not be a concern.

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CHAPTER 7.

GENERAL DISCUSSION AND CONCLUSIONS

Atherosclerosis is the most widespread vasculopathy in man. It can lead to a partial or total obstruction of an artery (stenosis). Surgical treatment of such a stenosis often leads to a secondary stenosis or restenosis. Both the primary and the secondary stenosis have in common that increased densities of vascular smooth muscle cells are found in the intima of the inflicted arteries. These smooth muscle cell-containing intimas are more thrombogenic than endothelial cells alone. Intimal smooth muscle cells produce extracellular matrix proteins and can cause fibrosis of the vessel wall. Migration of quiescent medial smooth muscle cells towards the damaged intima and proliferation within the intima are key processes in the development of both atherosclerosis and secondary stenosis.

In the first chapter of this thesis an outline is given of the processes involved in atherosclerosis and restenosis that occur in the vessel wall, and the role that smooth muscle cells play in these processes. Key processes are:

- the change from a quiescent medial smooth muscle cell phenotype to a proliferative synthetic state
- the migration of medial smooth muscle cells to the damaged intima of the blood vessel
- the subsequent proliferation of these neo-intimal smooth muscle cells.

In this chapter an outline is given of the role that cytokines, mechanical stress, and invasion of leukocytes play in the phenotypic changes and proliferation of smooth muscle cells, and therefore in the progression of atherosclerosis and restenosis.

Proteolytic enzymes such as plasminogen activators and matrix metallo proteinases are able to promote cell migration. This has been demonstrated in tumour cells and in several non-transformed cell types. Activation and inhibition of these proteolytic enzymes are therefore important in the regulation of cell migration.

In chapter two the mechanism of extracellular proteolysis involved in cell migration is reviewed. In particular the role that the plasminogen activator system and the metalloproteinases play in the migration of vascular smooth muscle cells is stressed. Many growth factors have an effect on the production of extracellular proteinases, their activators or their inhibitors and therefore affect SMC migration.

In the third chapter we assessed the production of components of the plasminogen activation system by vascular smooth muscle cells. Endothelial cells are well-known

producers of tissue-type plasminogen activator and the inhibitor PAI-1. Endotoxin for instance is known to increase plasminogen activator inhibitor activity in cultured endothelial cells and *in vivo* in circulation. The balance between plasminogen activation and inhibition is an important factor determining the ability of cells to migrate. It is therefore important to know the situation deeper within the vessel wall, especially regarding the contribution of vascular smooth muscle cells to the balance between plasminogen activation and inhibition. In this chapter we assessed the effect of bacterial lipopolysaccharide (endotoxin) on vascular smooth muscle cells. Vascular smooth muscle cells are potent producers of the main inhibitor of fibrinolysis: plasminogen activator inhibitor-1 (PAI-1) both *in vivo* in the vessel wall of experimental animals and *in vitro* in cultured rat vascular smooth muscle cells. The production of PAI-1 is increased by endotoxin *in vitro*, but also *in vivo*. Therefore smooth muscle cells, in addition to endothelial cells, are important contributors to the fibrinolytic/proteolytic balance within the vessel wall. The proteolytic activity within the vessel wall is thought to be an important factor that determines the possibility for cells to migrate through the vessel wall.

In the fourth chapter the effect was assessed of an extract from platelets and the main platelet factor PDGF on the production of extracellular proteinases of both the plasminogen-activation and the metallo proteinase systems and their inhibitors in rat aortic smooth muscle cell cultures. Platelet deposition at the site of injury in the vessel wall is thought to contribute to the migration of smooth muscle cells towards the intima. Platelet products such as PDGF are known to be chemotactic for vascular smooth muscle cells. In the process of smooth muscle cell migration extracellular proteases from the groups of the plasminogen activators and the metalloproteinases are involved. In this chapter, an effect of an extract from platelets and of the platelet factors PDGF and serotonin on the production of extracellular proteinases of both the plasminogen-activation and the metallo proteinase systems and their inhibitors in rat aortic smooth muscle cell cultures was demonstrated. This means that platelet activation can contribute to the local proteolytic balance in the vicinity of vascular smooth muscle cells. PDGF-BB activates both the plasminogen activation system (t-PA and PAI-1) and the metallo proteinase system (MMP-3 and TIMP-1). PDGF is therefore a key factor in the regulation of smooth muscle cell migration, not only as a chemoattractant, but also in its role as modulator of extracellular proteolysis.

Hypertension has since long been associated with an increased risk of cardiovascular diseases. Patients with a high renin profile, with a variant genotype of the angiotensin-converting enzyme and the angiotensin II-receptor 1 gene have a persistently increased risk even after normalisation of the blood pressure. Administration of ACE-inhibitors in several experimental animal models of atherosclerosis showed a reduction in the

formation of atherosclerotic lesions. ACE inhibitors also showed a reduction of neointimal proliferation in a rat balloon-induced injury model, although this could not be demonstrated in any other species. Angiotensin II is a vasoconstrictor and can induce hypertension. In addition this hormone has some other actions on cultured vascular smooth muscle cells, including the induction of smooth muscle hypertrophy and proliferation. PDGF is another vasoconstrictor, and in addition it is a mitogen for SMC and modulates the plasminogen activation and inhibition balance in cultured vascular smooth muscle cells, as was demonstrated in chapter 4. The local plasminogen activator activity is an important factor determining the ability of smooth muscle cells to migrate through the vessel wall. Angiotensin II shares with PDGF several steps in its signalling pathway and in chapter 5 we investigated whether angiotensin II was able to modulate the plasminogen activation in cultured rat aortic SMC similar to PDGF. Angiotensin II was indeed found to be a potent stimulus for the production of PAI-1 and t-PA. Moreover cultured SMC responded to the inactive precursor angiotensin-I with a similar increase in t-PA and PAI-1 production. From this we concluded that vascular smooth muscle cells have the ability to convert Ang I to the active product Ang II, without the help of endothelium cells that are known to express the angiotensin-converting enzyme.

In the sixth chapter we report a new method distinguishing between the plasminogen-dependent and an plasminogen-independent pathway to degrade extracellular matrix proteins. Smooth muscle cells are able to degrade radioactively labelled extracellular matrix proteins. In the absence of plasminogen the matrix degradation is reduced by 50%, when plasminogen was added to the cells full matrix degradation could be restored, the remaining 50% of the matrix degradation can not be inhibited by removing plasminogen from the culturing medium. We concluded that matrix degradation by SMC is partially mediated by plasmin, and partially by a plasmin-independent system. This was also demonstrated in another way. A labelled matrix was pre-digested with a plasmin preparation until all plasmin-sensitive material was removed. When rat aortic SMC were seeded on this pre-digested matrix additional degradation of matrix proteins was observed. We conclude that cultured smooth muscle cells use both mechanisms to degrade extracellular matrix material. This validates the findings of chapter four. The existence of these two parallel processes could have as consequence that when one of the two components is inhibited pharmacologically *in vivo*, the other may (partially) compensate this inhibitory effect. In other words it is possible that treatment of atherosclerosis or secondary stenosis through inhibition of smooth muscle cell migration is only effective (or more effective) if both proteolytic processes are inhibited.

Final conclusions.

The physiology of the vessel wall, especially in a pathological situation is a very complex one. Quiescent and activated endothelial cells and smooth muscle react in a

different manner, and they interact with invading monocytes and macrophages, and are modulated by factors from platelets and from the circulation. In this thesis we have focused on the role of just one player in the process: the smooth muscle cell. This is obviously a simplification, but a prerequisite for expanding the knowledge of the metabolism of the smooth muscle cell and of the etiology of atherosclerosis and restenosis. It implies that the utmost prudence is required in extrapolating any findings to the situation of the pathological vessel wall, and any findings need to be confirmed in animal studies, and certainly also in the clinic.

In this thesis we have added to the complex puzzle of the pathological vessel wall by further identification and characterisation of the proteolytic processes that smooth muscle cells use during the development of both atherosclerosis and secondary stenosis. Both are important areas of disease and of high clinical relevance. In view of the key role these proteolytic processes play in the pathology, high expectations exist for these processes as therapeutic targets. The study of these proteolytic processes in a model system of cultured vascular smooth muscle cells may bring us closer to that goal.

SAMENVATTING.

Atherosclerose is een aandoening die wordt gekenmerkt door plaques of laesies in de slagaderen. Deze aandoening veroorzaakt op den duur vernauwingen in de slagader, die kunnen leiden tot chronische aandoeningen zoals angina pectoris (pijn op de borst), claudicatio intermittens (etalagebenen) en multi-infarct dementie, maar ook acute aandoeningen zoals hart- en herseninfarcten. Atherosclerotische plaques kunnen chirurgisch worden behandeld door dotteren, waarbij het vernauwde deel van het bloedvat wordt opengerekt met een opblaasbaar ballonnetje, soms na de behandeling opengehouden door "stent", of door het plaatsen van een stukje donor bloedvat (een "by-pass") dat het bloed langs de vernauwing leidt. Na een tijd kan toch weer vernauwing van het bloedvat optreden. Deze restenose komt helaas in veel gevallen voor (30% van gedotterde patiënten). In dit proefschrift worden mechanismen onderzocht die betrokken zijn bij het ontstaan van de primaire atherosclerose, maar vooral bij de restenose die na chirurgische behandeling zo vaak optreedt.
Een gezonde slagader bestaat uit drie lagen: de buitenste is de adventitia die uit bindweefsel bestaat; de middelste of media bevat vrijwel alleen gladde spiercellen die de het bloedvat de stevigheid geven en het vermogen om samen te trekken; de binnenste laag of intima bestaat uit endotheelcellen die het bloedvat afscheiden van het stromende bloed en voorkomen dat bloedstolsels tegen de vaatwand afgezet worden. Als de binnenkant van een bloedvat om welke reden ook beschadigt, reageren gladde spiercellen en endotheel cellen hierop, en gaan delen om het defect weer te herstellen. Het is gebleken dat in de herstellende intima (de "neointima") vaak ook veel gladde spiercellen aangetroffen worden, die in de gezonde intima niet of nauwelijks worden aangetroffen. Na een paar cycli van schade en herstel kan van een atherosclerotische plaque worden gesproken. De situatie kan nog verslechterd worden indien de neointimale gladde spiercellen vetten en cholesterol opnemen, en als bloedplaatjes en ontstekingscellen lokaal groeifactoren uitscheiden. Bij restenose kan een soortgelijke reactie van de gladde spiercellen ontstaan, bewerkstelligd door zowel beschadigingen in de endotheellaag, groeifactoren uit bloedplaatjes en mechanische beschadigingen tijdens de operatieve behandeling van een atherosclerotische plaque. Er kunnen zich zoveel gladde spiercellen in de neointima ophopen dat deze een obstructie van het bloedvat veroorzaken. Het is dus belangrijk om de migratie van gladde spiercellen van de media naar de intima en de daarop volgende fase van celdeling te kunnen beheersen.
Bij celmigratie zijn vele factoren betrokken, maar extracellulaire proteolytische enzymen spelen een belangrijke rol. In de hoofdstukken 3 tot en met 5 worden factoren onderzocht die de balans tussen extracellulaire proteolyse activiteit en remming hiervan kunnen beïnvloeden. Endotoxine of bacterieel lipopolysaccharide is een stof die de balans tussen proteolyse en remming van proteolyse kan beïnvloedden, en in hoofdstuk 3 is aangetoond dat dit zowel in gekweekte rat aorta gladde spiercellen is aan te tonen,

als in vivo, in de aorta van ratten en konijnen, na injectie van endotoxine. Ook factoren uit een extract van bloedplaatjes kunnen de produktie van proteolytische factoren moduleren. De plaatjesfactor PDGF-BB stimuleert het plasminogeen activatie systeem (de synthese van t-PA en PAI-1) en van het metalloprotease systeem (MMP-3 en de remmer TIMP-1). Angiotensine II is een hormoon achtige stof die betrokken is bij het ontstaan van hoge bloeddruk, en in hoofdstuk 5 wordt beschreven dat deze stof ook de proteolytische balans, en dus het vermogen van gladde spiercellen om te migreren door de vaatwand beïnvloedt. In hoofdstuk 6 wordt een methode beschreven waarmee het bestaan van twee systemen van extracellulaire proteolyse betrokken bij de afbraak van extracellulaire matrix eiwitten door gladde spiercellen is aangetoond. Deze zijn het plasminogeen activatie systeem en de matrix metalloproteasen. Het bestaan van deze twee parallelle processen kan als consequentie hebben dat als in de kliniek beheersing van het restenose proces wordt nagestreefd door remming van de proteolytische enzymen, er op gelet moet worden dat de twee proteolytische systemen mogelijk in staat zullen zijn elkaars taak geheel of gedeeltelijk over kunnen nemen.

De processen die zich afspelen in de vaatwand, en zeker bij beschadiging en herstel zijn zeer complex. In dit proefschrift is de rol van slechts één speler, de gladde spiercel er uit gelicht. Deze simplificatie heeft het één en ander opgelicht over de rol van gladde spiercellen in migratie processen in de beschadigde vaatwand, en met name de betrokkenheid van proteolytische enzymen hierin. Gezien het belang van die celmigraties in de pathologie van de vaatwand bestaan er grote verwachtingen van het toepassen van remmers van extracellulaire proteolyse, die eerst met het beschreven model van gekweekte gladde spiercellen getest kunnen worden en later eventueel hun effectiviteit kunnen gaan bewijzen in de kliniek.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 22 augustus 1961 geboren in Lisse, waar hij ook zijn lagere school doorliep en in 1979 het diploma Atheneum-B aan het Fioretti College behaalde. In dat zelfde jaar begon hij met de studie biologie aan de Rijksuniversiteit te Leiden. De auteur heeft twee kandidaats examens biologie behaald in 1984, één met het hoofdvak biologie en één scheikunde als tweede hoofdvak. In 1984 en 1985 was hij student assistent bij de kursus histologie en embryologie en in september 1986 legde hij het doktoraal examen biologie af in Leiden. In het kader van vervangende dienstplicht werd de auteur in 1987 aangesteld bij het Gaubius Instituut van TNO te Leiden, waar hij onder leiding van Dr. J. Verheijen onderzoek deed naar struktuur-funktie relaties bij t-PA. Samenwerking van het Gaubius instituut met Prof. A. Maseri in Londen resulteerde in een aanstelling bij the Royal Postgraduate Medical School in 1990. Uit deze samenwerking onder begeleiding van Prof. C. Kluft is de basis gelegd voor het werk dat in dit proefschrift is beschreven. De groep van prof. Maseri is 1991 naar het Policlinico Gemelli in Rome overgegaan, waar de auteur het onderzoek heeft voortgezet bij het nieuw opgerichte Istituto di Cardiologia. Vanaf december 1994 is de auteur aangesteld als post-doc onderzoeker bij de afdeling huidziekten van het Academisch Medisch Centrum in Amsterdam waar hij onderzoek verricht naar de toepassing van huidvervangende biomaterialen bij huidtransplantaties.

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