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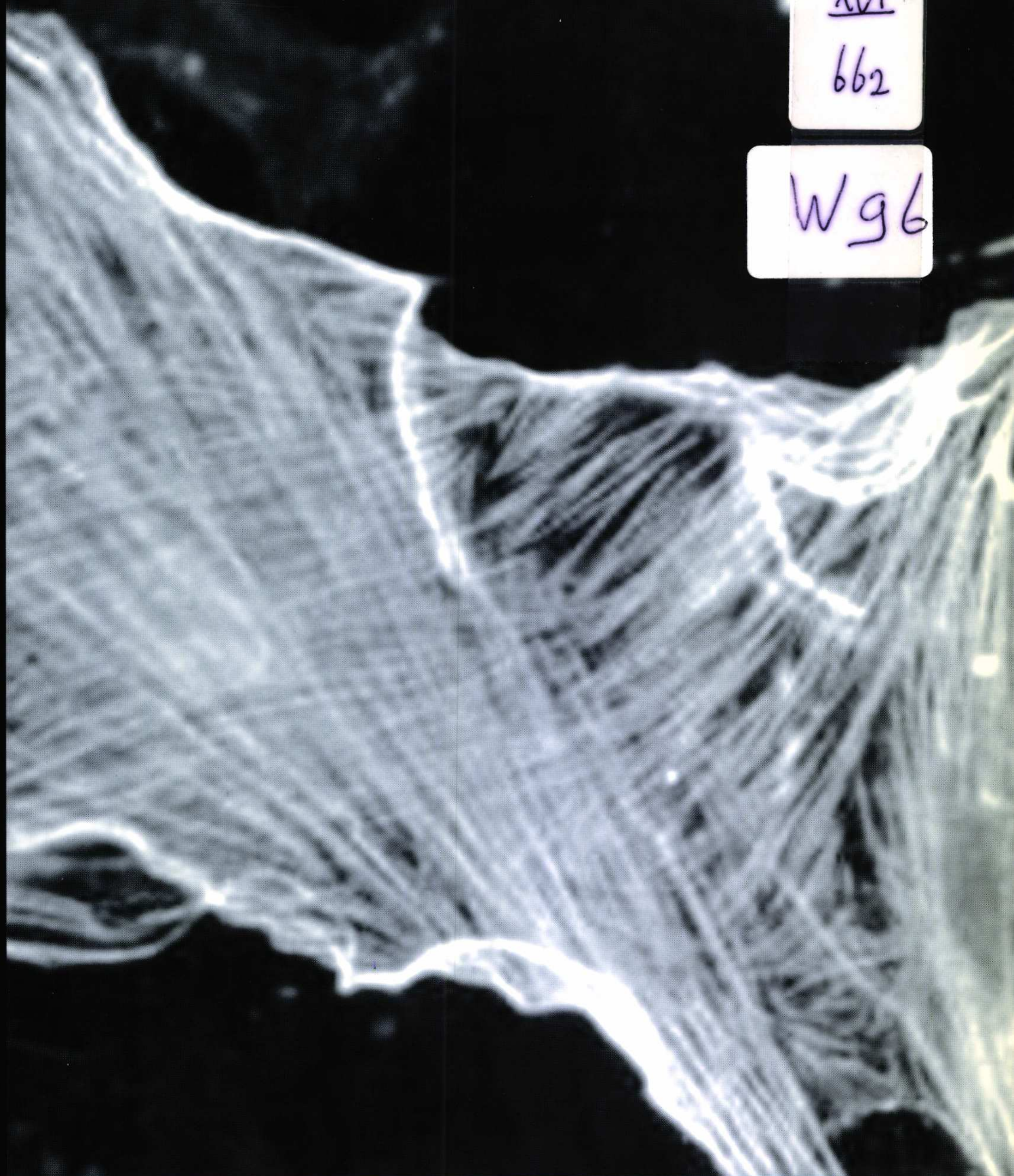
Monique Wijnberg



Migration of vascular cells and arteriosclerosis

Role of proteolytic processes

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Stellingen behorende bij het proefschrift:
Migration of vascular cells and arteriosclerosis. Role of proteolytic processes

1. Plasminogeen activatoren zijn in staat de migratie van humane gladdespiercellen *in vitro* te stimuleren *(dit proefschrift).*
2. De celdichtheids afhankelijke accumulatie van t-PA en PAI-1 in het medium van gladdespiercel kweken kan worden verklaard door een verschil in opname via leden van de LDL-receptor familie (*dit proefschrift*).
3. Gladdespiercellen die zijn geïsoleerd uit verschillende typen humane vaten vertonen een verschil in migratie gedrag (*dit proefschrift*).
4. De observatie dat laterale migratie van endotheelcellen niet plasmine afhankelijk is, terwijl de migratie van gladdespiercellen dat wel is, suggereert dat het remmen van plasmine activiteit de gladdespiercel migratie na PTCA zou kunnen voorkomen, zonder effecten op re-endothelialisatie (*dit proefschrift*).
5. Experimenten in u-PAR 'knock-out' muizen suggereren dat antiproteolytische therapie voor arteriële stenose gericht zou moeten zijn op de remming van u-PA *activiteit* en niet op u-PAR *binding* (*Carmeliet P. et al. J. Cell Biology 1998;140:233-245).*
6. Atherosclerose is een inflammatoire ziekte (*Ross R. New Eng J Med 1999;340:115-126*).
7. De rol die PAI-1 speelt in cellulaire migratie kan niet alleen verklaard worden op grond van zijn plasmine remmende activiteit (*Stefansson S. et al. TCM 1998;8:175-180*).
8. Het urokinase/uPAR/plasmine systeem heeft een dualiteit die het mogelijk maakt om enerzijds pericellulaire proteolyse en anderzijds cel-adhesie te reguleren (*Chapman HA. Current Opinion in Cell Biology 1997;9: 714-724*).
9. Het verrichten van wetenschappelijk onderzoek bij proefpersonen zonder het verkrijgen van een 'informed consent' is sinds 1 oktober een misdrijf en kan een onderzoeker een jaar gevangenisstraf opleveren (*Wet medisch-wetenschappelijk onderzoek met mensen, Staatsblad van het Koninkrijk der Nederlanden 26 februari 1998*).
10. In de huidige biomedische projecten wil men vaak datgeen bewijzen waarvan men al overtuigd is vóórdat de projectbeschrijving is geschreven (*MFM 1999:4:46-47*).

11. Het werken met gladdespiercellen wil niet zeggen dat experimenten met deze cellen altijd 'gladjes' verlopen.
12. Er is weinig verschil tussen corned-beef en een agressieve automobilist: beiden kunnen worden gezien als beest in blik.

Leiden, 17 november 1999 Monique Wijnberg

Migration of vascular cells and arteriosclerosis

Role of proteolytic processes

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 17 november 1999
te klokke 14.15 uur

door

Monique Janine Wijnberg
geboren te Alkmaar in 1971

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To study, to finish, to publish.
Benjamin Franklin, 1706-1790

Aan mijn ouders,
voor Peter.

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Chapter 1

General Introduction

Introduction

The term atherosclerosis refers to all of those lesions in which there is an increased thickness of the vessel wall, largely due to the accumulation of cells and connective tissue formed by these cells (1). In Western societies, atherosclerosis causes more deaths and chronic disorders via coronary artery disease, stroke and peripheral vascular disease than any other pathological process. In order to be able to devise methods to inhibit atherosclerosis, research into the factors and mechanisms involved in the process of atherosclerosis must be undertaken. In this introduction, some of the results of this research into atherosclerosis and restenosis will be discussed.

The normal vascular wall

The normal vessel wall consists of three layers: the inner intima (tunica intima) at the lumen of the vessel, the media (tunica media), and the outer adventitia (tunica externa) (2). The intima is composed of a single layer of endothelial cells and is separated from the media by an elastic membrane called the internal elastic lamina (lamina elastica interna). The latter is composed of laminin, collagen type IV, heparan sulphate proteoglycans and entactin (nidogen). The media consists of smooth muscle cell layers that are arranged in concentric and longitudinal layers and are embedded in an interstitial matrix, mainly composed of elastin (its main component), collagen (predominantly type I, type III, and type V), the remainder being mainly fibronectin and thrombospondin. The media is separated from the adventitia by the external elastic lamina (lamina elastica externa), composed of elastin and collagen. The adventitia is composed of collagen and elastin fibres and fibroblasts.

Atherosclerotic lesions

In contrast to the normal vascular wall, atherosclerotic lesions have a more diverse structure and have therefore clinically been classified into three different groups denoting their increasing severity: fatty streaks, intermediate/fibrofatty lesions, and fibrous plaques (1,3-5).

The fatty streak

Fatty streaks are small lesions consisting of an accumulation of lipid within macrophages and T-lymphocytes (foam cells) in the intima of the artery. Some smooth muscle cells in the intima can also contain lipid accumulations. Fatty streak formation itself is not associated with any risk of cardiovascular diseases and can be found in young and healthy people. It is thought that fatty streaks ultimately may be the precursors of atherosclerotic lesions.

The intermediate/fibrofatty lesion

The fibrofatty lesion consists of layers of lipid-filled macrophages and T-lymphocytes that alternate with layers of varying numbers of smooth muscle cells. They are surrounded by a connective tissue matrix of collagen, elastic fibres, and proteoglycans.

The fibrous plaque

A fully developed atherosclerotic plaque has a characteristic structure: a core containing necrotic debris, a-cellular lipid, and collagen encapsulated in the connective tissue at the base of the plaque, adjacent to the media. The plaque is covered with a fibrous connective tissue cap, which contains smooth muscle cells, macrophages and T-lymphocytes. Fully developed plaques are very heterogeneous in their composition. The relative amounts of lipid and collagen and the volume of the plaque made up by the core and the cap all vary widely. In advanced lesions calcification, necrosis, and thrombosis are observed. These lesions can occlude the vessel lumen completely and are often prone to rupture. They are potentially very dangerous because they can lead to e.g. myocardial infarction.

Atherosclerotic lesion development

Based on the animal and clinical studies that were known, Russell Ross formulated a hypothesis to explain how atherosclerotic lesions develop, the 'response-to-injury hypothesis' (1,3-5). In his hypothesis three phases of lesion progression are distinguished: fatty streak formation, plaque formation, and the advanced stages.

Fatty streak formation

Risk factors like high cholesterol levels, hypertension, diabetes, and cigarette smoking can cause injury to the endothelium. When this injury becomes chronic, it can lead to endothelial cell dysfunction. Excessive amounts of cholesterol-rich low-density lipoprotein (LDL) derived from the circulating blood accumulate within the intima. Endothelial cells start to express adhesion receptors for monocytes/macrophages and T-lymphocytes. These cells subsequently migrate between the endothelial cells under the influence of growth regulatory molecules and chemoattractants released by the endothelium, monocytes/macrophages, T-lymphocytes and smooth muscle cells. Macrophages and some intimal smooth muscle cells start to accumulate modified LDL. As a result of lipid accumulation this ingestion ultimately

leads to the formation of foam cells. Together with lymphocytes and intimal smooth muscle cells, the foam cells then form a fatty streak.

Plaque formation

Progression into a fibrofatty lesion and then to a fibrous plaque involves smooth muscle cell migration from the media to the affected area in the intima. Here smooth muscle cells start to proliferate, synthesise and secrete connective tissue matrix. This matrix consists of collagen, elastin, proteoglycans, and glycosaminoglycans. Breakdown of connective tissue and death of foam cells ultimately lead to the formation of a necrotic core.

Advanced stages

When the fibrous cap of the fibrous plaque has a uniform thickness and density, it provides stability to the plaque. However, when the fibrous cap is not uniform, and the 'shoulders' of the plaque are thin and macrophage-rich, the lesion may be unstable. In later stages unstable plaques may rupture or fissure, leading to the formation of a thrombus. This leads to further plaque growth and stenosis, or may even lead to acute symptoms, like myocardial infarction.

Restenosis

To be able to arrest the dangerous symptoms of atherosclerosis, vascular interventions have been deviced, including bypass surgery, percutanous transluminal balloon angioplasty (PTCA), atherectomy, and the application of vascular stents. However in 30-50% of treated patients restenosis (luminal renarrowing) of the vessel is induced within 3 to 6 months after surgery (6,7). Extensive research is and has been done to study the mechanisms that are involved in restenosis.

The most extensively studied animal model of the process of neointimal formation is the rat carotid artery balloon injury model (7-9). In this model a balloon catheter deendothelializes the carotid artery and stretches the media. After balloon injury three so-called 'waves' are distinguished. The first wave consists of medial smooth muscle cell replication in the injured vessel wall. This begins within 24 h and can last for a few days (0-3 days). The second wave then follows and smooth muscle cells cross the internal elastic lamina and form the neointima (3-14 days). Once smooth muscle cells have migrated into the intima, in a third wave, for some time (7 days-1 month) continuous cell division and extracellular matrix deposition follows. This latter process eventually accounts for the bulk of neointimal mass. Continued

stimulation of neointimal smooth muscle cell proliferation by mitogens may be important for the chronic accumulation of neointimal mass and is called restimulation or the fourth wave.
From Ross's 'response to injury' hypothesis and from animal models of arterial injury, restenosis is thought to involve hyperplasia of smooth muscle cells in the intima (6). So far there has, however, been a striking difference between animal models and humans in the efficacy of agents to prevent restenosis. Efforts with antiproliferative therapies, directed against smooth muscle cell replication have unfortunately not been proven to be of clinical benefit. This indicates that the mechanism of restenosis are still poorly understood and suggests that the mechanisms of intimal hyperplasia in animal models might be different from restenosis in human atherosclerotic vessels (7,10,11).

Smooth muscle cell phenotypes

An interesting aspect of the development of atherosclerosis and restenosis is that intimal smooth muscle cells are phenotypically different from medial smooth muscle cells (12-14). Characteristically, intimal cells have lower levels of contractile proteins and a decreased contractility. Furthermore they express a large number of proteins that may contribute to lesion development and/or stability. There are two main theories to explain their origin: (1) Intimal smooth muscle cells have altered their phenotype to a repair function after local injury. (2) Intimal smooth muscle cells are a population of phenotypically distinct smooth muscle cells that pre-exist in the normal blood vessel.
Campbell *et al.* (15,16) found that when adult media smooth muscle cells are put into culture they undergo extensive changes in their phenotype, and obtain many of the intimal smooth muscle cell characteristics. Their hypothesis is that smooth muscle cells are able to express a range of phenotypes with the 'contractile' and 'synthetic' states at the extreme ends. Contractile smooth muscle cells do not proliferate, and have a well-developed contractile apparatus. They respond to a wide variety of vasoconstrictor or vasodilator signals. The synthetic smooth muscle cells are in a mitotic state and have a less well-developed contractile apparatus. They have a rougher endoplasmatic reticulum, more Golgi and express genes for several growth-regulating factors and cytokines (17). On the basis of these morphological characteristics Campbell *et al.* proposed that medial smooth muscle cells have a 'contractile' phenotype that is modulated to a 'synthetic' phenotype when they start to migrate into the intima (15,16).
Another theory is that intimal cells arise from clonal expansion of a subpopulation of 'immature' cells present in the vessel wall media. This implies that at least two

phenotypically stable smooth muscle cell populations should exist. This hypothesis is supported by the demonstration that intimal smooth muscle cells are monoclonal in human atherosclerosis (18,19). Smooth muscle cell clones with apparent stable phenotypes in the rat have also been identified (11,20,21). Gabbiani *et al.* showed that cultured rat aortic smooth muscle cells exhibit at least two phenotypic variants: a 'spindle'-shaped phenotype, obtained from normal adult media, and a 'epitheloid' phenotype, obtained from intimal thickening after endothelial balloon injury. Both phenotypes can be cloned from each location, with media yielding a majority of spindle shaped clones and thickened intimas yielding a majority of epitheloid clones.

Proteolytic enzymes in the migration of cells

From the previous paragraphs it has become clear that the migration and proliferation of smooth muscle cells play essential roles in the formation of atherosclerosis and in restenosis. Migration of cells is a complicated process, involving several steps: adhesion to extracellular matrix components, localised degradation of pericellular matrix, de-adhesion from extracellular matrix, and movement into the modified matrix. A migrating cell penetrates its surrounding extracellular matrix by the focal degradation of extracellular matrix proteins by proteolytic enzymes. There are at least two enzyme systems, the plasminogen/plasmin system and the matrix metalloproteinases, which have been shown to play an important role in cell-migration processes that occur for example during wound healing, angiogenesis, and tumour metastasis (22-28). In the next paragraphs an overview of these enzyme systems and of their role in cellular migration is given.

The plasminogen activation system

While the plasminogen activation system was initially thought to be involved only in fibrinolysis (the dissolution of fibrin clots), it was later found to also play a role in cellular migration (29). The plasminogen activation system involves the following components:

Plasminogen/plasmin

Plasminogen is a single chain glycoprotein of approximately 92 kDa. Its concentration in plasma and interstitial fluids is 1-2 μM. Through the hydrolysis of an Arg-Val bond by plasminogen activators, the active serine proteinase plasmin is formed. It consists of a light and a heavy polypeptide chain connected by a disulphide bridge. The light chain (25 kDa)

contains the proteolytic domain, whereas the heavy chain contains five homologous 'kringle' structures of 10-12 kDa each (29,30).
Plasmin has a broad substrate specificity. In addition to fibrin, plasmin can directly cleave many extracellular matrix components like collagens, proteoglycans, laminin, fibronectin and vitronectin (29,31,32). It can also activate zymogens of the matrix-metalloproteinase class of enzymes, thereby expanding the proteolytic capacity (33). Plasmin also plays a role in the activation of latent growth factors, like TGF-β (35) and bFGF (FGF-2). Plasmin can be inhibited by α_2-antiplasmin and α_2-macroglobulin. When plasmin is cell-receptor bound it is protected against these inhibitors. A plasmin inhibitor that is frequently used in *in vitro* studies is bovine pancreas trypsin inhibitor, also known as aprotinin or Trasylol®.

Plasminogen activators

There have been two plasminogen activators identified, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (30,35). Like plasminogen, they belong to the serine-proteinase family.
u-PA was initially identified in urine. It is synthesised and secreted as an inactive single chain glycoprotein (pro-u-PA or scu-PA) of 54 kDa. Proteolytic enzymes (plasmin, kallikrein, and cathepsin) can cleave this zymogen into an active two-chain enzyme form (tcu-PA) (31). The aminoterminal A chain (33 kDa) consists of an epidermal growth factor (EGF) -like domain and a 'kringle' structure. The carboxy terminal B chain (21 kDa) consists of a catalytic domain. 54 kDa u-PA can also be cleaved by plasmin into a 33 kDa enzymatically active low molecular weight u-PA form (LMW-u-PA) and an aminoterminal fragment (ATF). LMW-u-PA consists of the intact B-chain and a small carboxyterminal portion (21 aminoacids) of the A-chain (29,31).
t-PA is secreted as a single chain glycoprotein of approximately 70 kDa. In contrast to u-PA, the single chain form of t-PA has catalytic activity, which does not differ much from the cleaved form. The single chain form can be converted to a two-chain form by plasmin, kallikrein or factor Xa. The two chains are kept together by a disulphide bridge. The A-chain of t-PA has a 'finger'domain a 'growth factor'domain and two 'kringles'. Through the finger and kringle 2 domain, t-PA is able to bind to fibrin, leading to stimulation of activity of t-PA (36,37). The B-chain has homology with u-PA and contains the catalytic centre (31).

Plasminogen activator inhibitors

The activity of the plasminogen activators can be inhibited by at least three specific serine proteinase inhibitors, the plasminogen activator inhibitors type 1 and 2 and proteinase-nexin I (30,38).

Plasminogen activator inhibitor-1 (PAI-1) is a single chain glycoprotein of 50 kDa. PAI-1 is the main plasminogen activator inhibitor in plasma. It has a high affinity for t-PA and the active forms of u-PA, but it does not bind to scu-PA. PAI-1 inhibits t-PA and u-PA by forming 1:1 SDS stable complexes with the catalytic domain. PAI-1 is synthesised in an active form, but after secretion by cells, is quickly transformed into a latent form. This latent PAI-1 can be reconverted into an active form by treatment with denaturants, negatively charged phospholipids or vitronectin. The latent form cannot inhibit plasminogen activators and does not form complexes with them. PAI-1 is often found in association with vitronectin. The binding of PAI-1 with vitronectin stabilises it in an active conformation and protects it from inactivation (30,39,40).

Plasminogen activator inhibitor-2 is found as an intracellular nonglycosylated form of 46 kDa and a secreted glycosylated form of 70 kDa (41,42). PAI-2 forms inactive complexes with u-PA and with two-chain t-PA, but is unable to bind to single chain u-PA or t-PA. PAI-2 is not so effective in the inhibition of plasminogen activators as PAI-1 (38,42). In contrast to PAI-1, PAI-2 does not bind to the extracellular matrix.

Proteinase-nexin I is a 43 kDa glycoprotein (38). It can inhibit several serine proteinases, like u-PA, t-PA, plasmin and thrombin. The activity of plasminogen activators is less than of PAI-1 or PAI-2.

Cellular receptors

There are several cellular receptors for both plasmin(ogen) and for the plasminogen activators described.

Plasmin(ogen) binds to cells with low affinity and high capacity via its lysine binding sites, which are localised in its kringle domains and recognise carboxyterminal lysines of cell-surface proteins (43-45). Annexin II and α-enolase are proteins with a carboxyterminal lysine that can function as plasminogen receptors (43,44,46,47). Also gangliosides have been found to bind plasmin(ogen) (43).

For t-PA, there are also several receptors known: annexin II (46,47), the mannose 6-phosphate receptor, enolase, and a 25 kDa no further characterised receptor on smooth muscle cells (48,49).

For u-PA, Vassali *et al.* first discovered a binding site on a monocytic cell line (31,50,51). This receptor is now known as the urokinase-receptor (u-PAR) and has been identified in many cell types (52). u-PAR is a 55-60 kDa glycoprotein, consisting of 3 homologous sequence repeats of which the aminoterminal domain (domain I) is the main domain engaged in u-PA binding (49). The receptor is anchored in the cell membrane with a glycosyl-phosphatidylinositol lipid (GPI) anchor. u-PA binds to u-PAR with high affinity (K_d 0.1-1 nM), via the EGF domain in the A-chain (31). Not only scu-PA and tcu-PA can bind to u-PAR, but also u-PA:PAI complexes and the aminoterminal fragment (ATF) (53,54). LMW-u-PA is, however, not able to bind because it lacks the EGF-domain. u-PA keeps its catalytic activity when bound to u-PAR but it is not protected from inhibition by PAI (55,56). u-PA:PAI complexes are rapidly cleared from the cell surface via endocytosis and degraded (57). This process involves members of the low-density lipoprotein (LDL)-receptor family.

The low-density lipoprotein (LDL)-receptor family

The most intensively studied members of the LDL-receptor family are the low-density lipoprotein receptor (LDL-receptor), the low-density lipoprotein receptor-related protein/ α_2-macroglobulin receptor (LRP), the very-low density lipoprotein receptor (VLDL-receptor), and epithelial glycoprotein 330 (gp330). Together these receptors play an important role in the catabolism of lipoproteins, proteinases, and proteinase-inhibitor complexes (58). Two of these receptors have been found in smooth muscle cells, namely LRP and the VLDL-receptor (59-61).

LRP

LRP is synthesised as a single chain precursor with a molecular mass of 600 kDa that is cleaved into a 515 kDa heavy chain and an 85 kDa light chain. Both subunits are held together by non-covalent interactions. The large subunit contains the ligand-binding domains. LRP is a highly conserved protein, which suggests an essential role. Indeed, targeted disruption of the LRP coding gene in mice demonstrated an essential role for this receptor during embryonic development (62). In normal arteries, LRP is found in smooth muscle cells in the media and vasa vasorum (59). Atherosclerotic arteries have a strong staining for LRP in intimal smooth muscle cells and in foam cells (61).

VLDL-receptor

The VLDL-receptor occurs in two forms: a 130 kDa (type I) and a 105 kDa (type II) form that differ only in the presence (type I) or absence (type II) of an O-linked carbohydrate domain. Both forms have the same binding affinity to several ligands (63,64). The VLDL-receptor is expressed by smooth muscle cells and endothelial cells in normal veins and arteries, as well as within atherosclerotic plaques. In the latter, VLDL-receptor is also expressed by macrophage-derived foam cells (60). Unlike LRP, homozygous VLDL-receptor knockout mice are viable and fertile (65).

Receptor-associated protein (RAP)

During purification of LRP, a 39 kDa glycoprotein termed receptor-associated protein (RAP) was identified. RAP binds with high affinity to LRP and VLDL-receptor and is able to block the binding of ligands to these receptors (63,66,67). Recently, RAP has been identified as a chaperon protein for LRP, required for the proper folding and processing of LRP during transport from the Golgi to the cell surface and for preventing the premature binding of co-expressed ligands (68-71).

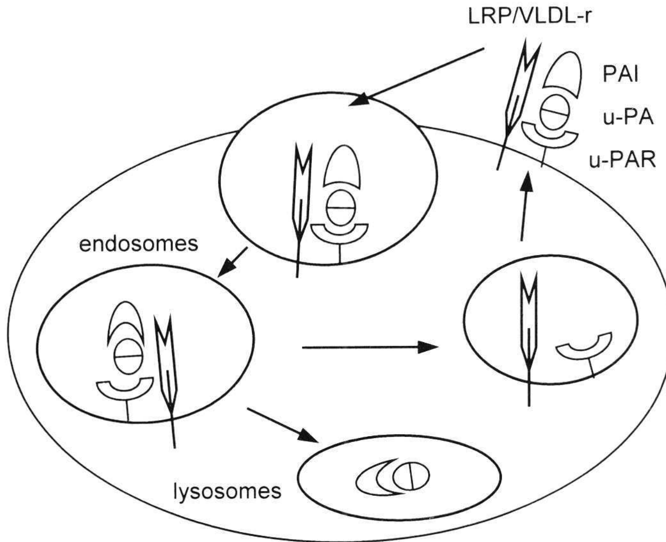

Figure 1. LDL-receptor-mediated uptake of u-PA:PAI-1 complexes

Regulation of plasminogen activator-activity by LRP and VLDL-receptor

By binding and internalisation of proteinase-inhibitor complexes, LRP and VLDL-receptor play a role in the regulation of proteinase activity (59). Among the proteinase and inhibitor complexes which are ligands for LRP and VLDL-receptor are t-PA, u-PA, and their complexes with PAI-1, PAI-2 and proteinase-nexin I (62,72-79). Scu-PA and tcu-PA bind to purified LRP with K_d values of 45 nM and 60 nM, respectively. These values are approximately 15 to 20-fold weaker than for the binding of the u-PA:PAI-1 complex to LRP (K_d=3 nM) (77). u-PAR does not internalise free u-PA. On the contrary, u-PAR-bound complexes of u-PA with PAI-1, PAI-2 or proteinase nexin-I are readily internalised in a process that requires binding to LRP or VLDL-receptor. In this process, u-PAR itself is also endocytosed, and after dissociation from its u-PA:inhibitor complexes, it recycles back to the cell surface (75,78,80-83) (figure 1).

The role of the plasminogen activation system in cellular processes

It was generally thought that t-PA is primarily involved in fibrinolysis and u-PA in pericellular proteolysis. However, in recent years evidence has accumulated that the plasminogen activators often play similar roles and can replace each other. Also other (non-proteolytic) functions have been discovered for the plasminogen activation system.

Localisation of proteolytic activity to the cell surface

Both plasminogen/plasmin, t-PA and u-PA can bind to the cell surface (48,84). Single chain u-PA can be converted to active u-PA by plasmin while it is receptor-bound and receptor-bound u-PA efficiently activates plasminogen. Binding of t-PA or u-PA and plasminogen to cell-binding sites strongly enhances plasminogen activation (45,85-89). Cell-bound plasmin is protected from inactivation by α_2-antiplasmin and α_2-macroglobulin, while plasmin in solution is quickly inactivated (43,85,86). In this way plasmin activity is restricted to the cell surface, thereby facilitating controlled pericellular matrix degradation (figure 2).

Signal transduction and mitogenesis

u-PA and t-PA have also been implicated in signal transduction processes. The signalling effects are, however, quite diverse between different cell types and include mitogenic effects, induction of migration, chemotaxis, and protein phosphorylation (49,90).

Cell adhesion

One of the steps involved in cell migration is adhesion to the matrix. This adhesion must not be so strong that it prevents movement, nor too weak to provide traction. Cells can adhere to the extracellular matrix via specific receptors called integrins. Chapman's group discovered that, besides the known vitronectin receptors (α_v-integrins), u-PAR can also function as a cellular adhesion receptor for the matrix protein vitronectin (91-93). u-PAR binds to vitronectin via its domain II and III, which is a binding site that is distinct from the u-PA binding site. Binding of u-PAR to vitronectin is further promoted by scu-PA, tcu-PA or u-PAR binding fragments of u-PA (92-94).

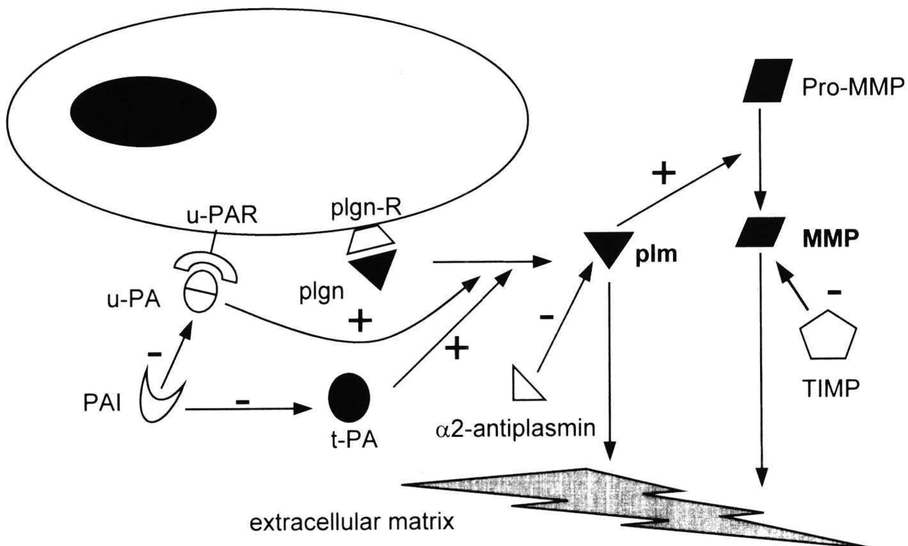

Figure 2. Plasminogen activator-mediated pericellular matrix degradation

Cell migration

Cellular migration is a very complicated process, in which the resulting migration capacity of cells is most likely highly dependent on the cell type, the extracellular matrix they have to migrate through, and the expression levels of members of the plasminogen activation system. Through the mechanisms described here, the plasminogen activation system may either promote or inhibit migration.

The Matrix metalloproteinase system

Similarly to the plasminogen activation system, the matrix metalloproteinase system is also thought to play an important role in cellmigration processes. The matrix metalloproteinase system consists of matrix metalloproteinases (MMPs) and their tissue inhibitors of metalloproteinases (TIMPs).

The matrix metalloproteinase (MMP) family can be divided into at least four main groups based on their substrate selectivities; the collagenases, the gelatinases, the stromelysins, and the membrane type MMPs (93-100). The collagenases (MMP-1, MMP-8, and MMP-13) cleave fibrillar collagens. The gelatinases (MMP-2 and MMP-9) are important in the breakdown of basement membranes and have a substrate specificity for denatured collagens (gelatins), types IV, V, VII, X, and XII collagens, vitronectin, and elastin. The stromelysins (MMP-3, MMP-7, MMP-10, and MMP-11) have broad substrate specificity. In addition to degrading extracellular matrix components, stromelysins can also activate other MMPs. A recently discovered group of MMPs is the membrane type MMPs (MT-MMPs) (103). There are at least four members of this group that all contain a membrane-binding domain. It is thought that they arrive at the cell-surface in an active form (100). Very recently, new members of the MMP-family were identified, MMP-18, MMP-19, and MMP-20 (104-106).

The MMPs can be inhibited by α_2-macroglobulin and by members of the tissue inhibitors of metalloproteinases (TIMP) family. To date four members of this family have been described. The TIMPs act specifically against the active forms of the MMPs and form 1:1 non-covalent complexes with them. TIMP-1 is a 29 kDa glycoprotein (96-98,100). In addition to forming complexes with active MMPs it also binds to proMMP-9. Interaction of TIMP-1 with activated MMP-9 results in proteinase inhibition, whereas interaction with proMMP-9 blocks MMP-3-mediated activation of the enzyme (98,101). TIMP-2 is a nonglycosylated 21 kDa protein (96-98). While TIMP-1 and TIMP-2 are secreted from cells and remain soluble, TIMP-3 binds to the connective tissue matrix (98,100). The most recently described member of the TIMP- family is TIMP-4 (107).

Expression of components of the plasminogen activation system and the matrix metalloproteinases in vascular smooth muscle cells

Several studies have demonstrated that cultured vascular smooth muscle cells produce both u-PA and t-PA as well as PAI-1 and PAI-2 (108-113). PAI-1 can be upregulated by several molecules such as the growth factors PDGF, EGF and TGF-β and cytokines like interleukin-1, TNF-α, and thrombin. PDGF and bFGF can increase t-PA, while there is no effect on u-PA

(113). The urokinase receptor has also been found on cultured smooth muscle cells and its expression can be upregulated by thrombin, EGF, TGF-β, bFGF, and PDGF (114).
In addition, smooth muscle cells have been shown to express several components of the metalloproteinase system. In cultures of human aortic medial smooth muscle cells, small amounts of MMP-1 and MMP-3 are produced. MMP-1 production can be increased by cytokines like PDGF, interleukin-1 and TNF-α (115-117). Synthesis of MMP-3 is increased in smooth muscle cells stimulated with interleukin-1 or TNF-α (116). Human arterial smooth muscle cells are able to produce MMP-9 after stimulation with interleukin-1 or TNF-α. (116). In contrast, MMP-2, TIMP-1 and TIMP-2 are constitutively expressed (116,118). Due to the diverse effects of regulatory molecules on expression of the various components, the overall effect on proteolytic activity in a particular system is often difficult to predict.

Outline and aim of the study

As discussed, migration of smooth muscle cells from the media towards the intima and subsequent proliferation in the intima are fundamental processes in the development of atherosclerosis and restenosis. One of the key steps in these processes is the proteolytic degradation of the extracellular matrix that encages smooth muscle cells.
One of the first suggestions that proteolytic enzymes play a role in smooth muscle cell migration came from the studies of Schleef and Birdwell who found that smooth muscle cell migration was strongly reduced when the cells were deprived of plasminogen or when the plasmin activity was inhibited (118). Reidy's group found that, following balloon injury, rat carotid arteries expressed both u-PA and t-PA. A marked increase in plasmin activity was detected 3 days after injury and was sustained for approximately another 10 days (119,120). When tranexamic acid, a compound that blocks plasmin activity, was added, there was a significant reduction in the rate of smooth muscle cell migration in ballooned arteries. After balloon injury, u-PA accumulated during the period of proliferation, whereas t-PA activity was increased at 4 days, around the time that smooth muscle cells started to migrate from the media to the neointima. Carmeliet and coworkers have generated transgenic mice that are deficient in components of the fibrinolytic system. They found that the rate and degree of neointima formation and neointimal cell accumulation after vascular injury were similar in wild-type, t-PA deficient and u-PAR deficient arteries. In contrast, both the degree and the rate of neointima formation in u-PA and plasminogen deficient mice were significantly reduced after injury, while neointima formation occurred earlier in PAI-1 deficient mice (121-127).

The subject of this thesis was to further identify the proteolytic enzymes and inhibitors that are involved in the process of migration and/or invasion of human vascular smooth muscle cells.

In **chapters 2 and 3** we describe studies on the role of the plasminogen activation system in smooth muscle cell migration using two *in vitro* models systems; lateral migration in a 'wound' assay, and invasion through a basement matrix (Matrigel®)-coated Transwell® system.

As discussed, it is thought that phenotypically different smooth muscle cells exist in the vessel wall. In **chapter 4**, plasminogen activation, proliferation and invasion of smooth muscle cells isolated from different sources of the human vasculature are examined.

Cell density can influence gene expression via cell-cell contacts between cells. In **chapter 5** we examined the effects of cell density on plasminogen activator and PAI-1 accumulation by human vascular smooth muscle cells.

In **chapter 6** we compare the role of the plasminogen activation system in endothelial cell and smooth muscle cell migration, and compare invasion and lateral migration models.

In **chapter 7** we compare the expression of components of the MMP system and the plasminogen activation system in the normal and atherosclerotic vessel wall.

Restenosis occurs usually focally in the treated vessel. Because local delivery would prevent unwanted side effects, therapy aimed at local delivery of restenosis inhibitors is being developed (128,129). One way to achieve local delivery of therapeutic agents is to transfect vascular cells with replication-deficient (adenoviral) viruses that encode factors, which can inhibit the restenotic process. In **chapter 8**, we show the effects of adenoviral transfer of genes that encode proteins that can influence the plasminogen activation system, in our *in vitro* smooth muscle cell migration model.

Finally, in **chapter 9** we discuss our results.

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Chapter 2

Urokinase and tissue-type plasminogen activator stimulate human vascular smooth muscle cell migration

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Summary

The objective of this study was to investigate the role of the plasminogen activation system in the migration of human vascular smooth muscle cells in vitro. After wounding of confluent human smooth muscle cell cultures by stripping cells away from their extracellular matrix, cells start to migrate from the wounded edge into the denuded area. Addition of plasmin to the culture medium resulted in an approximately 50% increase of migrated cells after 24 hours. The plasmin inhibitor aprotinin was able to reduce this effect to control levels. Migration could also be stimulated by addition of urokinase-type plasminogen activator (HMW-u-PA) (30%) or tissue-type plasminogen activator (t-PA) (28%). Simultaneous addition of aprotinin reduced this increase again to control levels, indicating that both HMW-u-PA and t-PA mediated plasminogen activation contributes to smooth muscle cell migration. Addition of low molecular weight u-PA (LMW-u-PA), a u-PA form lacking the receptor binding domain, or the aminoterminal fragment of u-PA (ATF), lacking the active site, had no effect on migration. These results suggest that cell surface bound u-PA activity is involved in stimulation of in vitro human smooth muscle cell migration.

Introduction

The migration of smooth muscle cells is thought to play an important role in intimal thickening in atherosclerosis and restenosis (1). In order to migrate, cells have to degrade the extracellular matrix surrounding them. This is accomplished through localised proteolysis of extracellular matrix components. Plasminogen activators are involved in a variety of cell migration and tissue remodelling processes (2,3). There are two physiological plasminogen activators; tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). For u-PA two molecular weight forms are described; a 54 kDa high molecular weight form (HMW-u-PA) and a 33 kDa low molecular weight form (LMW-u-PA). LMW-u-PA lacks the aminoterminal fragment (ATF) of HMW-u-PA and therefore cannot bind to the urokinase receptor (u-PAR) but is still enzymatically active. Both t-PA and u-PA can convert the zymogen plasminogen to the active serine protease plasmin. Plasmin is able to degrade a variety of extracellular matrix molecules and can activate latent metalloproteinases and growth factors. t-PA is thought to be primarily involved in fibrinolysis, whereas u-PA is believed to be more important in tissue remodelling and cell migration processes, especially when bound to its cell surface receptor (u-PAR) (2,3,4). Inhibition of u-PA activity reduces for example tumour cell migration (5,6), keratinocyte migration (7) and angiogenesis in a fibrin matrix (8).

In smooth muscle cell migration a role for the plasminogen activation system has been suggested from animal studies involving vascular injury (9,10,11). In this study, we demonstrate that plasminogen activation by either t-PA or u-PA can stimulate migration of smooth muscle cells in vitro, most likely by cell-surface located plasminogen.

Materials and methods

Cell Culture

Human umbilical vein smooth muscle cells (HUVSMC) were isolated from umbilical cords obtained from the department of obstetrics of the University Hospital Leiden, according to the guidelines of the Institutional Review Board of this hospital. After removal of endothelial cells (12), umbilical cord veins were incubated with isolation medium (Dulbecco's modified Eagle medium (DMEM), supplemented with 2 mmol/L glutamine, 10% (v/v) heat inactivated (30 min., 56°C) foetal calf serum (FCS) (Life Technologies, Paisley, Scotland), 100 μg/mL streptomycin, 100 U/mL penicillin and 0.75 g/L collagenase (Worthington Biochemical Corp., Freehold, NJ, USA). After 45 minutes the incubation medium containing detached cells was flushed from the veins. Cells were washed with culture medium (DMEM supplemented with 2 mmol/L glutamine, 10% (v/v) FCS, 100 μg/mL streptomycin and 100 U/mL penicillin) and grown on gelatine-coated culture dishes at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere.
Immunocytochemical analysis with a monoclonal antibody to smooth muscle α-actin (Boehringer Mannheim, Mannheim, Germany) (dilution 1:100) was used to characterise the isolated cells as smooth muscle cells. Cells were used for experiments between passage 4-6.

Migration assay

Confluent cultures of HUVSMC were mechanically wounded by detaching cells from their underlying extracellular matrix with Millipore filter strips (0.2 μm MF filters) (7). After wounding, cultures were washed with phosphate buffered saline (PBS) to remove residual detached cells. Subsequently, cultures were incubated in DMEM to which were added either, no additives, 0.15 μmol/L plasminogen (Biofine, the Netherlands), plasmin (Biofine, the Netherlands) (5 nmol/L), HMW-u-PA (Ukidan, Serono, Switzerland) (10 nmol/L), t-PA (Actilyse, Boehringer Ingelheim, Germany) (10 nmol/L) and/or aprotinin (500 K.I.U./mL), LMW-u-PA (Abbokinase, Abbot, USA) (10 nmol/L) or ATF (conditioned medium from LB6δF cells, a mouse fibroblast cell-line transfected with the aminoterminal domain of u-PA (5)) (0.1 μg/mL). Migration of cells into the wounded area was followed by means of phase contrast photo-microscopy and quantified by counting the number of cells that migrated into the denuded area after 24 hours from photographs taken along the edge of the wound.

Measurements on cell viability and growth

To determine the possible effects of HMW-u-PA or t-PA on cell growth, HUVSMC were seeded in 24 well plates. HMW-u-PA and t-PA were added to the cells in DMEM in the same concentrations as in the migration assay. After 24 hours the number of cells was determined with a heamocytometer.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed with Student's T-test. Statistical significance was accepted for $p<0.05$.

Results

The role of the plasminogen activation system in migration of vascular smooth muscle cells was studied by wounding confluent human umbilical vein smooth muscle cell (HUVSMC) cultures.

Role of plasmin in smooth muscle cell migration

Addition of plasmin (5 nmol/L) to the medium of wounded HUVSMC cultures resulted in a significant increase in the number of migrated cells (146 ± 12% of control conditions) (table 1). Simultaneous addition of aprotinin (500 K.I.U./mL) to the medium reduced the stimulatory effect of plasmin on migration to control levels (84 ± 8% versus control (100 ± 6%)) (table 1).

Control	100 ± 6%
Plasmin (5 nmol/L)	146 ± 12%*
Plasmin (5 nmol/L) + Aprotinin (500 K.I.U./mL)	84 ± 8%**

Table 1. The effect of plasmin on HUVSMC migration.

Wounded smooth muscle cell cultures were incubated in the absence or presence of 5 nmol/L plasmin with or without 500 K.I.U./mL aprotinin. The number of cells that migrated into the denuded area was determined after 24 hours from randomly taken photographs along the wounded edge and is expressed as percentage of control ± S.E.M. * different from control $P<0.003$ (n=10) ** different from plasmin $P<0.0004$ (n=10).

Role of plasminogen activator in smooth muscle cell migration

In another set of experiments we added plasminogen to the HUVMC medium without (control) or with HMW-u-PA (10 nmol/L) or t-PA (10 nmol/L). The addition of HMW-u-PA or t-PA also resulted in a stimulation of migration of HUVSMC (130 ± 9% and 128 ± 8% of control respectively) (table 2). The stimulatory effect of these plasminogen activators on smooth muscle cell migration could be diminished to control levels by the simultaneous addition of aprotinin (500 K.I.U./mL) to the culture medium (table 2).

Role of u-PA/u-PA receptor in smooth muscle cell migration

We also studied the effect of LMW-u-PA which lacks the aminoterminal fragment of HMW-u-PA and therefore cannot bind to the urokinase receptor and of the aminoterminal fragment of

HMW-u-PA (ATF) which is able to bind to u-PAR but has no proteolytic activity. The addition of LMW-u-PA (10 nmol/mL) or ATF (0.1 μg/mL) to the HUVSMC medium was not able to stimulate migration (96 ± 9% and 99 ± 9% of control respectively) of HUVSMC.

Control	100 ± 10%
HMW-u-PA (10 nmol/L)	130 ± 9%*
HMW-u-PA (10 nmol/L) + Aprotinin (500 K.I.U./mL)	95 ± 7%**
t-PA (10 nmol/L)	128 ± 8%*
t-PA (10 nmol/L) + Aprotinin (500 K.I.U./mL)	74 ± 8%***
LMW-u-PA (10 nmol/L)	96 ± 9%
ATF (0.1 μg/mL)	99 ± 9%

Table 2. The effect of HMW-u-PA, t-PA, LMW-u-PA and ATF on HUVSMC migration. Wounded smooth muscle cell cultures were incubated in the presence of 0.15 μmol/L plasminogen and in the absence (control) or presence of 10 nmol/L HMW-u-PA or t-PA with or without 500 K.I.U./mL aprotinin or in the presence of 10 nmol/L LMW-u-PA or 0.1μg/mL ATF. The number of cells that migrated into the denuded area was determined after 24 hours from randomly taken photographs along the wounded edge and is expressed as percentage of control ± S.E.M. * different from control $P<0.05$ (n=10), ** different from HMW-u-PA $P<0.01$ (n=10), *** different from t-PA $P<0.0001$ (n=10).

Effect of u-PA and t-PA on smooth muscle cell proliferation

u-PA and t-PA have been shown to have a mitogenic effect on several cell-types (13,14,15). Herbert et al. (16) noticed that t-PA had a mitogenic effect on human aorta smooth muscle cells. To exclude that the effects of HMW-u-PA or t-PA on the number of cells counted in the migration assay where due to a proliferative effect of these plasminogen activators, HUVSMC were cultured in the presence of plasminogen with or without t-PA or HMW-u-PA. After 24 hours, cell numbers were determined. Neither t-PA nor HMW-u-PA had a significant effect on cell number compared to control conditions (results not shown). Therefore the effects observed in cell migration seem not to be due to differences in proliferation rate, indicating that HMW-u-PA and t-PA mediated plasminogen activation are most likely directly involved in human smooth muscle cell migration in vitro.

Discussion

The data presented in this study demonstrate that plasmin mediated proteolysis can contribute to smooth muscle cell migration (table 1) and that this plasmin can be generated either by u-PA or t-PA mediated activation of plasminogen (table 2) because the effects of HMW-u-PA and t-PA on cell migration could be diminished by the plasmin inhibitor aprotinin. The observation that both

LMW-u-PA, lacking the receptor binding domain, and ATF, lacking the protease domain, have no effect on migration suggests that cell surface bound u-PA is essential for smooth muscle cell migration. An interesting observation is that human vascular smooth muscle cells express u-PAR and that in response to wounding in vitro, smooth muscle cells polarise and concentrate cell surface u-PAR to their leading edge, thereby probably facilitating directional migration (17).
These results are in close agreement with in other data. An involvement of plasmin activity in vitro was shown by Schleef and Birdwell, who found that bovine aorta smooth muscle cell migration was strongly reduced when plasminogen was depleted from the culture medium (18). Clowes et al. (9) observed that after balloon injury in the rat carotid artery u-PA was rapidly induced, followed by a slower induction of t-PA. In the same model the addition of tranexamic acid, an inhibitor of plasminogen activation, resulted in a reduction of migration of smooth muscle cells (10), indicating that the generation of plasmin is necessary for the migration of smooth muscle cells. Experiments with u-PA, t-PA and PAI-1 'knock-out' mice (11) showed that after vascular trauma, deficiency of t-PA does not affect the degree or rate of neointima formation, but that deficiency of PAI-1 significantly accelerates and deficiency of u-PA greatly reduces and delays neointima formation.
The presence of compounds of the plasminogen activation system has been shown in human atherosclerotic lesions (19) and suggests that plasminogen activation could also be involved in human vascular remodelling. The present study indicates that both t-PA and u-PA can contribute to human smooth muscle cell migration in vitro, probably via the activation of plasminogen. For the stimulation of migration by u-PA both activity and binding to the cell-surface receptor seems involved.

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Chapter 3

The migration of human smooth muscle cells in vitro is mediated by plasminogen activation and can be inhibited α_2-macroglobulin receptor associated protein

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Summary

The plasminogen activation system is thought to be important in cell migration processes. A role for this system during smooth muscle cell migration after vascular injury has been suggested from several animal studies. However, not much is known about its involvement in human vascular remodelling. We studied the involvement of the plasminogen activation system in human smooth muscle cell migration in more detail using an in vitro wound assay and a matrix invasion assay. Inhibition of plasmin activity or inhibition of urokinase-type plasminogen activator (u-PA) activity resulted in approximately 40% reduction of migration after 24 hours in the wound assay and an even stronger reduction (70-80%) in the matrix invasion assay. Migration of smooth muscle cells in the presence of inhibitory antibodies against tissue-type plasminogen activator (t-PA) was not significantly reduced after 24 hours, but after 48 hours a 30% reduction of migration was observed, whereas in the matrix invasion assay a 50% reduction in invasion was observed already after 24 hours. Prevention of the interaction of u-PA with cell surface receptors by addition of soluble u-PA receptor or a_2-macroglobulin receptor associated protein (RAP) to the culture medium, resulted in a similar inhibition of migration and invasion. From these results it can be concluded that both u-PA and t-PA mediated plasminogen activation can contribute to in vitro human smooth muscle cell migration and invasion. Furthermore, the interaction between u-PA and its cell surface receptor appears also to be involved in this migration and invasion process. The inhibitory effects on migration and invasion by the addition of RAP suggests an involvement of a RAP sensitive receptor of the LDL receptor family, possibly the LDL-receptor related protein (LRP) and/or the VLDL receptor.

Introduction

Mechanical injury of the vessel wall induces migration and proliferation of smooth muscle cells, eventually leading to vessel wall thickening and restenosis (1,2). For migration cells have to degrade the extracellular matrix in which they are embedded. This is accomplished by localised proteolytic degradation of the extracellular matrix surrounding the cells. One of the systems involved in proteolysis of the extracellular matrix is the plasminogen activation system. It is known to be involved in a wide range of cell migration and tissue remodelling processes (3), including vessel wall remodelling in angiogenesis (4,5) and restenosis (6).

The plasminogen activation system consists of a cascade of enzymes ending with the zymogen plasminogen. This can be converted to the active serine protease plasmin by two plasminogen activators, tissue type plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA). Both plasminogen activators can be inhibited by plasminogen

activator inhibitor type-1 (PAI-1) and type-2 (PAI-2). t-PA is thought to be primarily involved in fibrinolysis whereas u-PA would be primarily involved in tissue remodelling and cell migration processes (7). u-PA can bind to a specific cellular receptor (u-PAR) that is anchored in the plasma membrane by its glycosyl phosphatidyl inositol (GPI) anchor (8,9). u-PAR not only localises the proteolytic activity of u-PA to the cell surface but is also involved in the internalisation of u-PA:PAI complexes (10,11) and in signal tranduction (12-14). Internalisation of u-PA:PAI complexes, most likely after they are bound to u-PAR, is mediated by various members of the LDL receptor family, such as the LDL receptor related protein/a_2-macroglobulin receptor (LRP), glycoprotein 330 (gp330) and the VLDL receptor (15-20). By influencing the internalisation of u-PA and t-PA, receptors of the LDL receptor family can indirectly influence cell surface plasminogen activation by removing these compounds or their PAI complexes from the cell surface.

Plasmin can degrade a broad range of extracellular matrix molecules and can activate latent metalloproteinases or growth factors. Inhibition of u-PA activity reduces tumour cell migration (21,22), keratinocyte migration (23) and angiogenesis in a fibrin matrix (24). The plasminogen activation system is thought to be also involved in vascular remodelling. In vitro, an involvement of plasmin activity was shown by Schleef and Birdwell (25) who found that bovine aortic smooth muscle cell migration was strongly reduced when plasminogen was depleted from the culture medium. Clowes et al. (6) observed a rapid induction of u-PA and a slower induction of t-PA after balloon injury in the rat carotid artery. In the same animal model, Jackson and Reidy (26) showed that tranexamic acid, a potent inhibitor of plasminogen activation, could strongly reduce neointima formation, a process involving smooth muscle cell migration. In recent experiments with plasminogen, t-PA, u-PA and u-PAR 'knock-out' mice it was shown that especially plasminogen and u-PA are important in vascular remodelling (27,28). Furthermore, the presence and regulation of components of the plasminogen activation system in human atherosclerotic lesions (29-33) suggests an involvement of this system in human vascular remodelling in vivo. Recently it has been shown that addition of exogenous HMW-u-PA as well as t-PA can induce human smooth muscle cell migration in vitro (34). Evidence for a role of the endogenous plasminogen activation system in migration of human smooth muscle cells however, is lacking.

In the present study, we demonstrated the direct involvement of the endogenous plasminogen activation system in migration of human smooth muscle cells in vitro. Both u-PA and t-PA mediated plasminogen activation as well as the interaction of u-PA with its receptor appears to be involved in the regulation of human smooth muscle cell migration in vitro. In addition, a

role for LRP and/or the VLDL receptor in this process is suggested by the inhibitory effect of RAP.

Materials and methods

Cell Culture

Human umbilical vein smooth muscle cells (HUVSMC) were isolated from umbilical cords. After removal of endothelial cells (35), umbilical cord veins were incubated with isolation medium (Dulbecco's modified Eagle medium (DMEM), supplemented with 2 mmol/L glutamine, 10% (v/v) heat inactivated (30 min., 56°C) foetal calf serum (FCS) (Life Technologies, Paisley, Scotland), streptomycin (100 μg/mL), penicillin (100 U/mL) and 0.75 g/L collagenase (Worthington Biochemical Corp., Freehold, NJ, USA). After 45 minutes the incubation medium containing detached cells was flushed from the veins. Cells were washed with culture medium (DMEM supplemented with 2 mmol/L glutamine, 10% (v/v) FCS, streptomycin (100 μg/mL) and penicillin (100 U/mL)) and grown on gelatine-coated culture dishes at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere.
Human artery smooth muscle cells (HASMC) were isolated from specimens of the left internal mammary artery. Left internal mammary arteries were kindly provided by Prof. Huysmans of the department of Thorax Surgery of the Academic Hospital Leiden, Leiden, the Netherlands after approval by the local ethical committee. Adhering fat and connective tissue were removed, and the adventitia and endothelial cells were scraped off with a scalpel blade. The media was minced into 1-2 mm pieces, and medial smooth muscle cells were isolated with the explant method (36) in culture medium containing additional 10% (v/v) heat inactivated (30 min., 56°C) human serum (local blood bank) on gelatine-coated culture dishes. After 2-3 weeks, cells began to migrate from the explants. When cells reached confluence, they were subcultured in culture medium.
Immunocytochemical analysis with a monoclonal antibody to smooth muscle a-actin (asm-1, Boehringer Mannheim, Mannheim, Germany) was used to characterise the isolated cells as smooth muscle cells. Cells were used for experiments between passage 3-6.

Migration assay

Confluent cultures of HUVSMC and HASMC were mechanically wounded by detaching cells with Millipore filter strips (0.2μm MF filters) in order to leave the underlying extracellular matrix in the denuded area intact (23). Wound edges were marked by scratching the culture dish with a scalpel blade. After wounding, cultures were washed with phosphate buffered saline (PBS) to remove residual detached cells. Subsequently, cultures were incubated in culture medium to which either no additives (control), the plasmin inhibitor aprotinin 100 KIU/mL (Trasylol, Bayer AG, Leverkussen, Germany), rabbit polyclonal antibodies neutralising human t-PA (37) (125 μg/mL) or u-PA (38) (125 μg/mL) or rabbit control IgG (125 μg/mL) were added. Cells were also incubated in culture medium containing 2 μg/mL human soluble u-PAR purified by affinity chromatography on urokinase-Sepharose from conditioned medium of transformed CHO cells, kindly provided by Dr. U. Weidle (39), or 5 μg/mL RAP produced in E.Coli strain DH5a as a fusion protein with glutathione S-transferase (RAP-GST) and purified by glutathione-Sepharose chromatography as described previously (40). Migration of cells into the wounded area was followed by means of phase contrast photo-microscopy and quantified from photographs taken along the edge of the wound by counting the number of cells that had

migrated into the denuded area after 24 and 48 hours. In all cases nine randomly selected fields were counted. The results were expressed as mean ± S.E.M.

Invasion assay

Invasion of HUVSMC was assayed using a Transwell system with Matrigel coated polycarbonate filters (8 µm pore size, Costar, Cambridge, MA). Matrigel (Collaborative Biomedical Products, Bedford, MA) was layered onto the filter (14 µg/well) and allowed to dry at room temperature. The membrane was hydrated with DMEM before use. Cultured HUVSMC were trypsinised and suspended at a concentration of 4×10^5 cells/mL in DMEM supplemented with 0.15 µmol/L plasminogen. A volume of 100 mL of cell suspension was placed in the upper chamber, and a volume of 600 mL of DMEM supplemented with 0.15 µmol/L plasminogen and 10% (v/v) FCS as chemoattractant was placed in the lower chamber. To both lower and upper chambers either aprotinin (100 KIU/mL), anti u-PA IgG (125 µg/mL), anti t-PA IgG (125 µg/mL) or no additives (control) were added. After 24 hours of incubation at 37°C in 5% CO_2/95% air (v/v) the cells were fixed with 2.5 % (v/v) glutaraldehyde. Matrigel and cells on the upper side of the filter were scraped off with a cotton swab, and cells on the lower side of the filter were stained with 2 g/L crystalviolet. Invasion was determined by counting the cells detected in microscopic fields (magnification x 200, n=24).

Effects on cell viability and growth

To determine possible mitogenic or cytotoxic effects of the various additives soluble u-PAR, RAP, aprotinin and antibodies to u-PA and t-PA, cell viability and growth of HUVSMC was analysed in the absence or presence of these additives.

To determine cell growth, HUVSMC were seeded in 24 well plates and grown to 80% confluency. Compounds were added to the cells in fresh culture medium in the same concentrations as in the migration assay. After 24 hours the number of cells was determined with a heamocytometer. To measure cell viability, $1\text{-}2 \times 10^3$ cells were seeded per well in a gelatine coated 96-well plate. After 24 hours, compounds were added to the cells in fresh culture medium in the same concentrations as used in the migration assay. After an additional incubation period of 48 hours, culture medium was replaced with 1 g/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO, USA) in DMEM, followed by a 3 hour incubation at 37°C (41). The MTT solution was removed, and the formed formazone was dissolved in DMSO or 2-propanol. The optical density was measured at 540 nm using a microtiterplate reader (Titertek, MultiSkan).

Fibrin underlay

Fresh culture medium was added to confluent HUVSMC cultures, and media samples were taken after 0, 24 and 48 hours. These samples were subjected to polyacrylamide gel electrophoresis under non-reducing conditions with a 10% (w/v) running, 5% (w/v) stacking gel according to Laemlli (42). After electrophoresis, gels were washed in 2.5% (v/v) Triton X-100 to remove sodium dodecyl sulphate (SDS) and placed on a plasminogen containing fibrin/agarose layer as described by Granelli-Piperno and Reich (43). Upon incubation plasminogen activators appear as clear lysis zones on an opaque background. Molecular weights were derived from the migration distance of human t-PA and u-PA standards electrophoresed in parallel lanes.

Assay for u-PA and t-PA antigen

The amounts of u-PA and t-PA in conditioned medium of HUVSMC were measured using specific enzyme linked immunosorbent assays for u-PA (38) and t-PA (Imulyse-kit, Biopool).

Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference test. Statistical significance was accepted for $p<0.05$.

Results

Expression of plasminogen activators by human smooth muscle cells

Human umbilical vein smooth muscle cells (HUVSMC) synthesise t-PA and u-PA. Fibrin zymography revealed plasminogen activator:PAI complexes but no free plasminogen activators in media samples obtained after 24 and 48 hours of incubation (figure 1), whereas with immunoassay production levels of 27±2 ng t-PA/(10^6 cells, 24 hrs) and 2.8±0.4 ng u-PA/(10^6 cells, 24 hrs) were found in the conditioned media of HUVSMC.

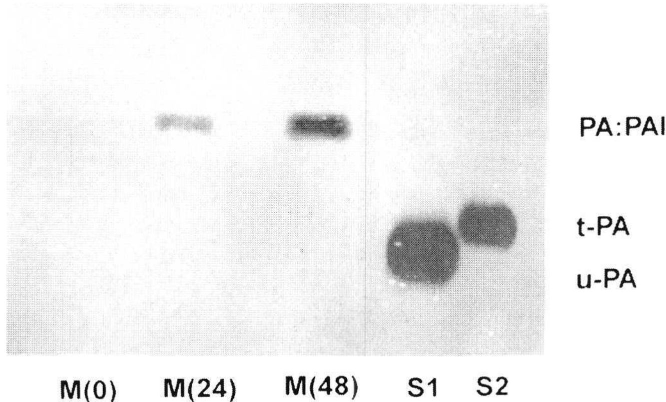

Figure 1. Fibrin zymography of human umbilical vein smooth muscle cell (HUVSMC) media
Media samples of confluent HUVSMC were obtained after 0 hours (M0), 24 hours (M24) and 48 hours (M48) and analysed for the presence of plasminogen activators as described under methods. As standards human u-PA (S1) and t-PA (S2) were used.

Involvement of u-PA and t-PA in human smooth muscle cell migration

Migration into the wounded area of confluent cultures was followed under various conditions for 48 hours to evaluate the role of plasmin and plasminogen activators (figure 2). A marked

reduction of the number of cells that migrated into the denuded area was observed after addition of the plasmin inhibitor aprotinin (100 KIU/mL) to the culture medium. Inhibition of migration was also observed after the addition of antibodies neutralising u-PA or t-PA to the culture medium. Control IgG had no effect (not shown).

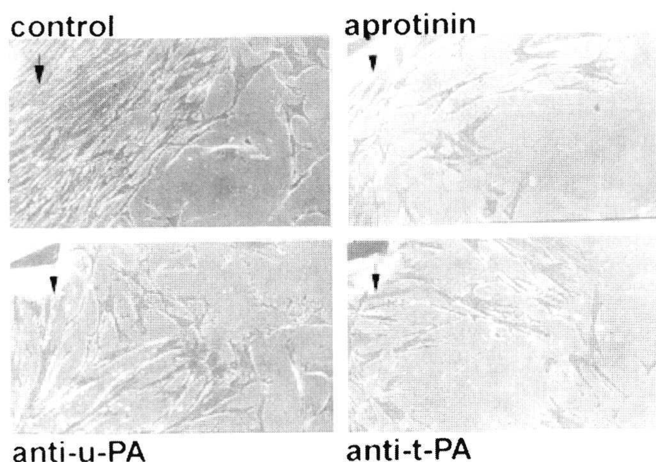

Figure 2. Migration of HUVSMC
Confluent cultures of HUVSMC were wounded and subsequently incubated in the absence or presence of 100 KIU/mL aprotinin or rabbit polyclonal antibodies to human u-PA or t-PA (125 μg/mL). Migration of cells into the denuded area was monitored by phase contrast photomicroscopy. Representative photographs of migrated HUVSMC after 48 hours are shown (original magnification x260). The original edge of the denuded area is indicated with an arrow.

Migration of HUVSMC was quantified 24 and 48 hours after wounding by counting the number of cells that migrated into the denuded area (figure 3). Aprotinin added to the culture medium inhibited migration to 62±7% after 24 hours and 61±5% after 48 hours compared to control cultures. Antibodies to u-PA reduced the number of migrated cells to 58±6% of control after 24 hours and 58±3% of control after 48 hours. Antibodies to t-PA however, had no significant inhibitory effect after 24 hours, but inhibited the migration of HUVSMC after 48 hours to 69±6%. Control IgG had no effect on migration (not shown).
Similar experiments were performed with human smooth muscle cells isolated from the left internal mammary artery (HASMC) (figure 4). After 48 hours aprotinin significantly reduced the number of migrated cells to 80±4% of control while antibodies to u-PA and t-PA reduced it to 69±3% and 78±5% of control respectively.

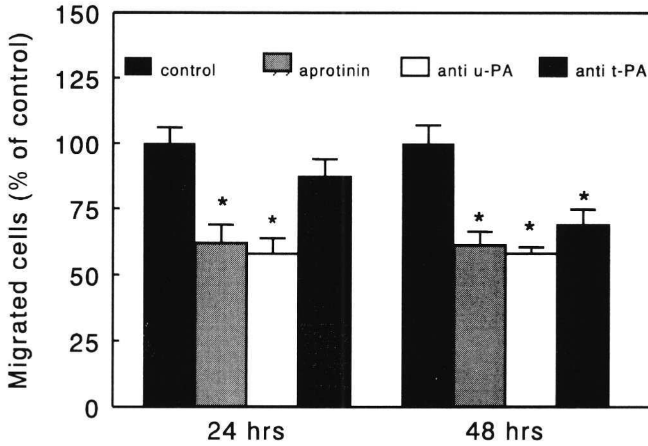

Figure 3. Quantification of HUVSMC migration
Wounded cultures were incubated in the absence or presence of 100 KIU/mL aprotinin, anti-u-PA IgG, anti-t-PA IgG (125µg/mL). The number of cells that migrated into the denuded area was determined after 24 and 48 hours from consecutive photographs along the edge of the wound and is expressed as percentage of control ± S.E.M. * $P<0.001$ (n=9).

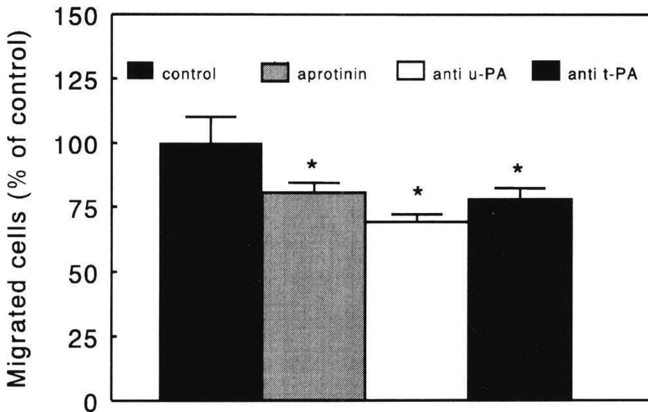

Figure 4. Quantification of human aorta smooth muscle cell migration
Wounded cultures were incubated in the absence or presence of 100 KIU/mL aprotinin, anti-u-PA IgG (125µg/mL) or anti-t-PA IgG (125 µg/mL). The number of cells that migrated into the denuded area was determined after 48 hours from consecutive photographs along the edge of the wound and is expressed as percentage of control ± S.E.M. * $P<0.05$ (n=9).

Involvement of receptor-bound u-PA in human smooth muscle cell migration

Binding of u-PA to its cellular receptor has been shown to be an important step in cell migration e.g. in tumour cells (7,21,22). The effect of the interaction of u-PA with its receptor on the migration of smooth muscle cells was studied, using soluble u-PAR. This protein, which lacks the GPI anchor, competes for the binding of u-PA to its cell surface u-PAR. Addition of soluble u-PAR (2 μg/mL) to the medium of wounded HUVSMC cultures inhibited smooth muscle cell migration (figure 5A). Quantitative analysis showed a significant inhibition of the number of migrated cells to 58±8% of control after 24 hours and 64±7% of control after 48 hours (figure 5B). Similar results were obtained with HASMC, in this case there was a reduction to 76±5% of control after 48 hours of the number of migrated cells.

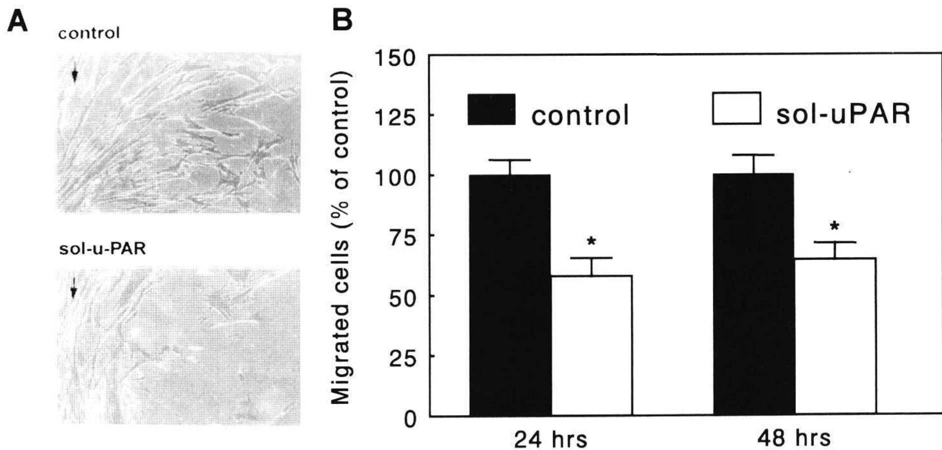

Figure 5. Influence of interaction of u-PA and its receptor on the migration of HUVSMC
Confluent cultures of HUVSMC were wounded and subsequently incubated in the absence or presence of 2 μg/mL soluble u-PAR. Migration of cells into the denuded area was monitored by phase contrast photomicroscopy. Representative photographs of migrated HUVSMC after 48 hours are shown (original magnification x260) (**A**). The original edge of the denuded area is indicated with an arrow. The number of cells that migrated into the denuded area was determined after 24 and 48 hours from consecutive photographs along the edge of the wound and is expressed as percentage of control ± S.E.M. * P<0.001 (n=9) (**B**).

Involvement of members of the LDL receptor family in human smooth muscle cell migration

Recently, it became apparent that internalisation of u-PA:PAI-1 complexes bound to u-PAR can be mediated by various members of the LDL receptor family, such as LRP, the VLDL receptor and gp330. Since LRP and the VLDL receptor are expressed in smooth muscle cells (15-20), they may play a role in u-PA mediated smooth muscle cell migration. To study the possible involvement of these receptors in smooth muscle cell migration we used a 39 kDa protein, the receptor associated protein (RAP), a chaperone protein that copurifies with LRP and is able to block the binding of ligands to members of the LDL receptor family, including the interaction of u-PA:PAI-1 complexes to LRP and the VLDL receptor (17).

The involvement of LRP and/or the VLDL receptor in smooth muscle cell migration in vitro was investigated by adding RAP-GST (5 μg/mL) to wounded cell cultures, and analysed as described above. Addition of RAP-GST significantly inhibited the number of migrating cells to 61±8% of control after 24 hours and 52±5% of control after 48 hours (figure 6B). Similar results were obtained for HASMC, were addition of RAP-GST inhibited migration to 77±8% of control after 48 hours (results not shown). Since RAP-GST was produced in E.Coli it inevitably contains endotoxin which could complicate the results. However, exogenous added endotoxin (lipopolysaccharide,10 μg/mL) had no effect on migration (not shown).

Invasion of human smooth muscle cells

Since smooth muscle cells in vivo have to migrate predominantly through rather than over an extracellular matrix, we examined whether plasmin, u-PA, t-PA, soluble u-PAR and RAP-GST also play a role in invasion through an extracellular matrix barrier. Smooth muscle cell invasion was determined after 24 hours in a Transwell system by quantifying the number of cells on the lower side of Matrigel-coated filters. Aprotinin reduced the number of cells invading through Matrigel to 20±3% of control (figure 7A). Antibodies to u-PA inhibited smooth muscle cell invasion to 32±5% of control, while antibodies to t-PA inhibited invasion to 53±3% of control (figure 7A). Soluble u-PAR reduced the number of invaded cells to 52 ± 4% of control (figure 7A). RAP-GST was able to inhibit invasion to 65 ± 8% of control (figure 7B).

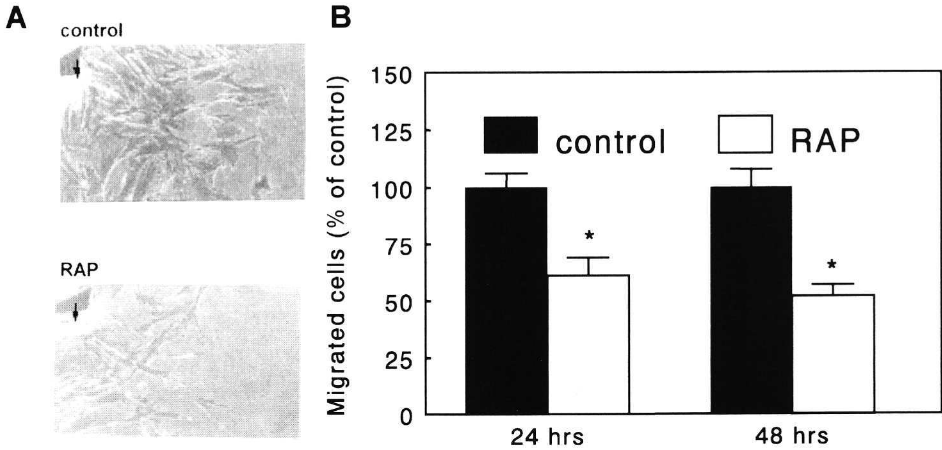

Figure 6. Role of LRP or the VLDL receptor on the migration of HUVSMC
Confluent cultures of HUVSMC were wounded and subsequently incubated in the absence or presence of 5 μg/mL RAP. Migration of cells into the denuded area was monitored by phase contrast photomicroscopy. Representative photographs of migrated HUVSMC after 48 hours are shown (original magnification x260) (**A**). The original edge of the denuded area is indicated with an arrow. The number of cells that migrated into the denuded area was determined after 24 and 48 hours from consecutive photographs along the edge of the wound and is expressed as percentage of control ± S.E.M. * $P<0.001$ (n=9) (**B**).

Cell growth and viability of human smooth muscle cells

To exclude the possibility that the inhibition of migration or invasion by aprotinin, soluble uPAR, RAP or antibodies to u-PA and t-PA was due to a toxic or antiproliferative effect, HUVSMC were cultured in the presence of these additives. After 24 hours of incubation, cell numbers were determined by counting with a heamocytometer. After 48 hours of incubation, cell viability was determined by staining with MTT. None of the compounds used had a significant effect on cell number (figure 8A) or cell viability (figure 8B). Therefore the effects observed are not based on an inhibition of proliferation or cytotoxicity.

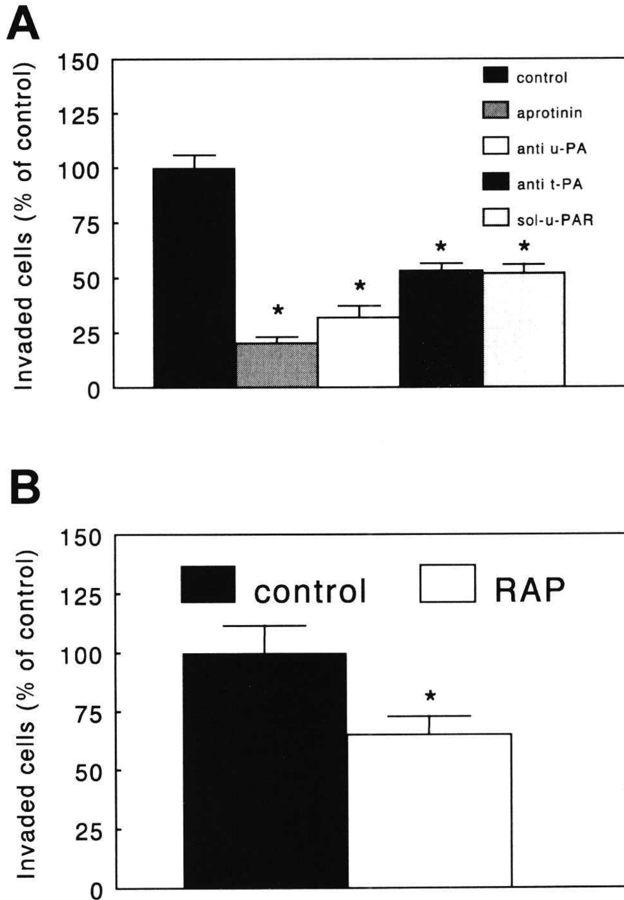

Figure 7. Effect of aprotinin, antibodies to u-PA or t-PA, soluble u-PAR or RAP-GST on invasion of HUVSMC through a matrix barrier
Transwell filters were coated with Matrigel. Smooth muscle cell suspension was placed in the upper chamber and DMEM containing chemoattractant (10% (v/v) FCS) was placed in the lower chamber. To both chambers 100 KIU/mL aprotinin, 125 μg/mL anti-u-PA IgG, 125 μg/mL anti-t-PA IgG, 3 μg/mL soluble u-PAR or no compound (control) was added (**A**). In another experiment to both chambers 5 μg/mL RAP-GST or no compound (control) was added (**B**). After 24 hours invasion was measured as described in methods and expressed as percentage of control ± S.E.M. *$p<0.001$ ($n=24$).

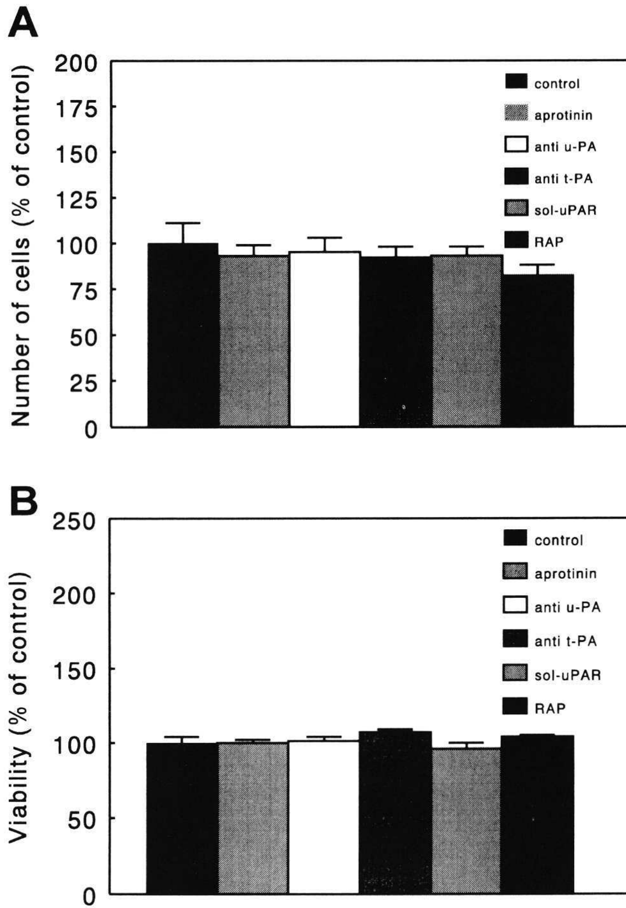

Figure 8. Cell growth and viability of HUVSMC

Cell growth was determined by incubating subconfluent smooth muscle cell cultures in the absence or presence of 100 KIU/mL aprotinin, 125 μg/mL anti-u-PA IgG, 125 μg/mL anti-t-PA IgG, 2 μg/mL soluble u-PAR or 5 μg/mL RAP for 24 hours. Cell numbers were determined with a heamocytometer (A). Results are expressed as percentage of control cell numbers ± S.E.M. (n=3). Cell viability was determined by seeding 1-2x10^3 cells/mL in gelatine coated 96 well plates. After 48 hours cell viability was determined by MTT staining (B). Results are expressed as percentage of control absorbance (540 nm) ± S.E.M. (n=10).

Discussion

Previously we have shown that human smooth muscle cell migration is induced by the addition of HMW-u-PA or t-PA (34). Here we report that in wounded cultures of HUVSMC, inhibition of plasmin and u-PA reduced the number of migrated cells with 40% after 24 and 48 hours. Inhibition of t-PA showed no effect after 24 hours but after 48 hours there was a 30% reduction in the number of migrated cells. Similar observations were made for HASMC although the extent of inhibition was somewhat lower in this case. With invasion through Matrigel even stronger inhibitory effects of aprotinin and neutralising antibodies recognising u-PA and t-PA were discerned. The observed differences in migration seem not to be due to cytotoxic effects or differences in proliferation rate. This indicates that u-PA and t-PA mediated plasminogen activation are most likely directly involved in human smooth muscle cell migration in vitro, probably through plasmin mediated proteolysis. The previously observed stimulation of cell migration by adding u-PA or t-PA was also completely inhibited by inhibition of plasmin (34).

Our results with human smooth muscle cells are in close agreement with data reported for animal experiments. It has been shown by Clowes et al. (6) that in vivo after balloon injury in the rat carotid artery the expression of u-PA is rapidly induced, after a few days followed by an induction of t-PA. The addition of tranexamic acid, an inhibitor of plasminogen activation, resulted in reduction of migration of smooth muscle cells (26). Also in our in vitro experiments u-PA appears to be mostly involved in the early migration phase (24 hrs in figure 3) whereas t-PA effects were observed in the later phase (48 hrs in figure 3). In a recent analysis of neointima formation after vascular trauma in 'knock-out' mice it was observed that deficiency of t-PA or PAI-1 does not affect the degree or rate of neointima formation, but that deficiency of plasminogen or u-PA greatly reduces and delays neointima formation (27,28). These data suggest that at least in mice, u-PA rather than t-PA contributes to vascular wound healing and the development of restenosis.

We found only partial inhibition of smooth muscle cell migration by inhibition of the plasminogen activation system. It was observed by Sperti et al. (44) that degradation of extracellular matrices by rat aorta smooth muscle cells occurs only partly via a plasmin mediated mechanism. These results suggest a role for other proteases in smooth muscle cell migration. Indeed, matrix metalloproteinases have been shown to contribute to the migration potential of smooth muscle cells (45,46).

It has been demonstrated that binding of u-PA to its cell surface receptor u-PAR stimulates matrix degradation and migration of tumour cells (21,22). Okada et al. (47) showed that human vascular smooth muscle cells express u-PAR and that in response to wounding in vitro

smooth muscle cells polarise and concentrate u-PAR at the cell surface u-PAR of their leading edge, thereby probably facilitating oriented migration. In atherosclerotic lesions an increased expression of u-PAR has been observed (29). We show that migration and invasion of smooth muscle cells is markedly reduced in the presence of soluble u-PAR, a compound preventing the binding of u-PA to its cell surface receptor. This implies that smooth muscle cell migration is at least in part mediated by receptor bound u-PA. Recently, it has been shown that u-PA and u-PAR might also be involved in cell-matrix interactions via binding to vitronectin (48). This may be an additional mechanism involved in cell migration.

LRP and the VLDL receptor, members of the LDL receptor family, have been shown to play a role in the endocytosis and subsequent degradation of many ligands, including u-PAR bound u-PA:PAI-1 complexes (15,16,18). The turnover of inhibited u-PA could be an important mechanism for the regeneration of unoccupied u-PAR and thus regulate the cell surface bound u-PA activity. In this way turnover of u-PA could also be involved in cell migration. Smooth muscle cells express LRP and the VLDL receptor (unpublished observations, 15-18). Addition of RAP-GST, an antagonist for binding of ligands to members of the LDL receptor family, inhibiting the internalisation of u-PA:PAI complexes (15,16), was able to inhibit migration and invasion of HUVSMC. This suggests a role for LRP and/or the VLDL receptor in smooth muscle cell migration.

In summary our data indicate that for human smooth muscle cells, u-PA but also t-PA mediated plasminogen activation is involved in human smooth muscle cell migration and invasion in vitro. Furthermore two types of receptor, u-PAR and LRP and/or the VLDL receptor appear to be involved, suggesting that plasmin formation at the cell surface is a critical factor in the migration process.

Acknowledgements

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Chapter 4

Differences in invasion between human smooth muscle cells from umbilical vein, saphenous vein and internal mammary artery; relation to the expression of the plasminogen activation system

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Submitted

Summary

Smooth muscle cells can express a range of phenotypes. Smooth muscle cells from intimal thickenings are phenotypically different from media smooth muscle cells. Migration and proliferation are important processes involved in the development of intimal thickening. Several studies have demonstrated the role of the plasminogen activation system in the migration and proliferation of smooth muscle cells. In this study we determined the proliferation and invasion capacity of smooth muscle cells that were isolated from three different vessels; saphenous vein, internal mammary artery, and umbilical vein. Whereas we found no differences in proliferation, we show that there are clear differences in the expression of t-PA, u-PA, and PAI-1 and subsequent differences in plasminogen activator activity between the smooth muscle celltypes. Whereas u-PA activity was low in all three celltypes studied, t-PA activity was lowest in UV-SMC (0.25 IU/10^6cells), and higher in SV-SMC (0.8 IU/10^6cells) and IMA-SMC (1.2 IU/10^6cells). These findings could in part explain the differences we found in the invasion capacity of the smooth muscle celltypes. Whereas the invasion of SV-SMC could be inhibited by an inhibitor of plasmin (aprotinin), the invasion of UV-SMC could not. Furthermore, the invasion of UV-SMC could be stimulated by active t-PA or u-PA, whereas the invasion of SV-SMC could not. The results for IMA-SMC were, however, more variable. These findings together suggest that phenotypically different smooth muscle cells exist between different vessels, and may explain some of the differences in the ability of vessels to develop intimal thickening.

Introduction

Migration and proliferation of smooth muscle cells are important processes contributing to intimal thickening, which occurs after vascular injury and during the development of atherosclerosis. One of the factors involved in smooth muscle cell migration is the tightly controlled degradation of the extracellular matrix that surrounds smooth muscle cells. Several proteolytic enzymes and inhibitors are known to be produced by smooth muscle cells, amongst which are the plasminogen activators t-PA (tissue-type plasminogen activator) and u-PA (urokinase-type plasminogen activator) and their main physiological inhibitor PAI-1. Both t-PA and u-PA activate the inactive pro-enzyme plasminogen to plasmin. In addition to its ability to degrade fibrin, plasmin can degrade several extracellular matrix molecules, like laminin, fibronectin, vitronectin and proteoglycans. Furthermore, plasmin can activate latent metalloproteinases, which are another group of proteolytic enzymes expressed by smooth muscle cells and known to be involved in cellmigration (1,2). Several studies have

demonstrated that the plasminogen activation system is involved in vascular smooth muscle cell migration (3-7).

Smooth muscle cells are heterogeneous and can express a spectrum of different phenotypes. At the end of the spectrum are the contractile and the synthetic phenotype. The contractile phenotype is thought to represent the normal medial smooth muscle cell, whereas the synthetic phenotype is thought to represent the (neo)intimal smooth muscle cell (8,9). Some studies have shown that not only do phenotypically distinct smooth muscle cells exist within one vessel but that there are also differences in smooth muscle cell phenotype between vessels (10,11).

The variability of vessels to develop intimal thickenings after vascular injury may be (partly) explained by a difference in smooth muscle cell phenotype. In this study we therefore examined the proliferation and invasion capacity of *in vitro*-cultured smooth muscle cells that were isolated from different types of human vessel: saphenous vein, internal mammary artery, and umbilical vein. Because the plasminogen activation system has been shown to play an important role in smooth muscle cell migration, we determined the expression levels of plasminogen activators and PAI-1, and their role in the invasion of the smooth muscle cell-types.

Materials and Methods

Cell Culture

All vessels used in this study were obtained from the Academic Hospital Leiden, the Netherlands, according to the guidelines of the Institutional Review Board of this hospital.

Human saphenous vein smooth muscle cells (SV-SMC) were isolated from unused pieces of saphenous vein which were obtained after by-pass surgery. After the endothelial cells were gently scraped off, the adventitia was carefully stripped from the media. The media was then cut into small fragments (1-2 mm). SV-SMC were either isolated using the explant method (12), or by incubating media fragments overnight with 0.075% (w/v) collagenase (Worthington, Biochemical Corp. Freehold, NJ, USA) in culture medium: Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Paisley, Scotland), supplemented with 5% (v/v) heat-inactivated (30 min, 56°C) foetal bovine serum (FBS, Life Technologies, Paisley, Scotland), 5% (v/v) heat-inactivated human serum (local Blood Bank) and penicillin (100 U/mL)/streptomycin (100 µg/mL) (Biowhittaker, Verviers, Belgium).

Human internal mammary artery smooth muscle cells (IMA-SMC) were isolated by the explant method (12,13) from specimens of the left internal mammary artery.

Human umbilical vein smooth muscle cells (UV-SMC) were isolated from umbilical cords. After the removal of endothelial cells (14), umbilical cord veins were incubated with isolation medium (DMEM, 10% (v/v) FBS, penicillin (100 U/mL)/streptomycin (100 µg/mL), and 0.75 g/L collagenase (Worthington Biochemical Corp., Freehold, NJ, USA)). After 45 minutes, the isolation medium containing detached cells was flushed from the veins and cells were washed with culture medium.

All smooth muscle celltypes were grown on gelatin (Merck, Darmstadt, Germany) coated dishes at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere. The culture medium was replaced every 3-4 days and subcultures were established by trypsin/EDTA treatment of confluent layers at a split ratio of 1:3. Smooth muscle cell isolations were used for experiments between passages 2-14.

Proliferation curves

Smooth muscle cells were seeded in a 1:3 split ratio in 6-well plates and grown under normal culture conditions. At 1-13 days after seeding, cells were harvested and counted in a Bürker-Türk chamber. Counts were performed in duplicate wells and were used to determine growth curves. Regression coefficients were calculated from the exponential part of the growth curves with the curve fit option of SPSS 8.0.

Enzyme-linked immunosorbent assays

Assay of u-PA antigen was performed as described (15). t-PA antigen was determined with the Thrombonostika t-PA ELISA (Organon Teknika, Boxtel, The Netherlands), and PAI-1 antigen with IMULYSE^{TM} PAI-1 (Biopool, Umeå, Sweden).

Plasminogen activator activity

Spectrophotometric assays of plasminogen activator activity in cell-conditioned media were performed as described by Verheijen *et al.* (16). Media were incubated in the presence of CNBr-digested fibrinogen fragments and Glu-plasminogen. Plasmin generation was assessed by monitoring the cleavage of the chromogenic substrate H-D-Val-L-Leu-L-Lys-p-nitroanilide (S-2251, Chromogenix) in a spectrophotometer at a wavelength of 405 nm. The inhibition of plasmin generation by the addition of 40 µg/mL rabbit anti-human t-PA IgG (17) in the assay was used to calculate t-PA, and the inhibition of plasmin generation by the addition of 100 µmol/L amiloride in the assay was used to calculate u-PA activity.

Inactivation of u-PA and t-PA

5 µmol/L HMW-u-PA (Ukidan, Serono, Switzerland) in 0.1 mol/L Tris-HCl pH 7.5, 0.1% (v/v) Tween-80, 1 mol/L NaCl was incubated with 100 µmol/L D-Glu-Gly-Arg-Chloromethylketone (GGACK, Calbiochem, La Jolla, CA, USA) for 1 h at room temperature. Thereafter the solution was put over a Sephadex G50 fine column and the run-through was collected. t-PA (Actilyse, Boehringer Ingelheim, Germany) was inactivated in the same way with D-Phe-Pro-Arg-Chloromethylketone (PPACK, Calbiochem, La Jolla, CA, USA). Proper inactivation of u-PA was checked with L-pyroGlu-Gly-L-Arg-p-nitroanilide (S-2444, Chromogenix) as substrate, and proper inactivation of t-PA was determined with H-D-Ile-L-Pro-L-Arg-p-nitroanilide (S-2288, Chromogenix) as substrate.

Invasion assay

Invasion of smooth muscle cells was assayed using Matrigel® (Collaborative Biomedical Products, Bedford, MA). Matrigel® is a basement membrane matrix extract derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma (18). Matrigel® was diluted in serum-free culture medium (DMEM with penicillin (100 U/mL)/streptomycin (100 µg/mL)) and 37.5 µg/well was layered onto pre-wetted Transwell polycarbonate filters (8 µm pore size, Costar, Cambridge, MA). It was allowed to dry overnight at room temperature and was rehydrated with serum-free culture medium before use.

Smooth muscle cells were incubated in serum-free culture medium overnight. Cells were trypsinised and washed twice with DMEM, penicillin (100 U/mL)/streptomycin (100 μg/mL), 10% (v/v) FBS to inactivate the trypsin. Cells were suspended at a concentration of 2 x 10^5 cells/mL in serum-free culture medium supplemented with 0.15 μmol/L Glu-plasminogen. A volume of 200 μL cell suspension was placed in the upper chamber, and a volume of 900 μL DMEM, penicillin (100 U/mL)/streptomycin (100 μg/mL) supplemented with 0.15 μmol/L Glu-plasminogen and 10% (v/v) FBS as chemoattractant was placed in the lower chamber. To both lower and upper chambers either aprotinin (100 KIU/mL) (Bayer, Leverkusen, Germany), HMW-u-PA (10 nmol/L), GGACK-inactivated HMW-u-PA (10 nmol/L), t-PA (10 nmol/L), PPACK-inactivated t-PA (10 nmol/L), or no additives (control) were added. After 42-144 hours of incubation (depending on the invasion rate of the cells) at 37°C in 5% CO_2/95% air (v/v), the cells were fixed with 2.5 % (v/v) glutaraldehyde. Matrigel® and cells on the upper side of the filter were scraped off with a cotton swab, and cells on the lower side of the filter were stained with 2 g/L crystalviolet. Invasion was determined by measuring the surface area covered by the cells in 12 microscopic fields (magnification 100x) per well with image analysis software (Optimas). Incubations were performed in duplicate and at least three different smooth muscle cell isolations per type of vessel were tested.

Statistical analysis
All data are presented as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference test. Statistical significant differences were accepted for $p<0.05$.

Results

Growth properties of human smooth muscle cells isolated from different vessels

We first measured the number of cells per well in confluent layers (95-100% of surface area covered with cells) of human smooth muscle cells that were isolated from umbilical vein (UV), saphenous vein (SV), or internal mammary artery (IMA). We noticed that UV-SMC had the largest number of cells (4.80 ± 0.25 x 10^5 cells/well (n=114, mean ± S.E.M.)). SV-SMC and IMA-SMC had about 2 times less cells/well (2.06±0.14 x 10^5 cells/well (n=45) and 2.11±0.12 x 10^5 cells/well (n=63)).

To measure the growth rate of smooth muscle cells, confluent cell-cultures were seeded in a 1:3 split ratio in 6-well plates and grown under normal culture conditions. Cell counts were performed between 1-8 days after seeding (figure 1). Most of the cell-isolations became confluent (95-100% of the tissue culture well covered with cells) between 5-8 days after seeding. When the smooth muscle cell-layers became confluent, their growth rates started to decline. From the exponential part of the growth curves slopes were calculated that were taken as a measure of growth rates. There was some variability in growth rate, both between cellcultures from the same type of vessel (but from different patients) and from different types of vessels. The average growth rate among the smooth muscle celltypes was however not

significantly different. The slopes were for UV-SMC 0.414 ± 0.075 (n=5), for SV-SMC 0.390 ± 0.056 (n=3), and for IMA-SMC 0.303±0.068 (n=3).

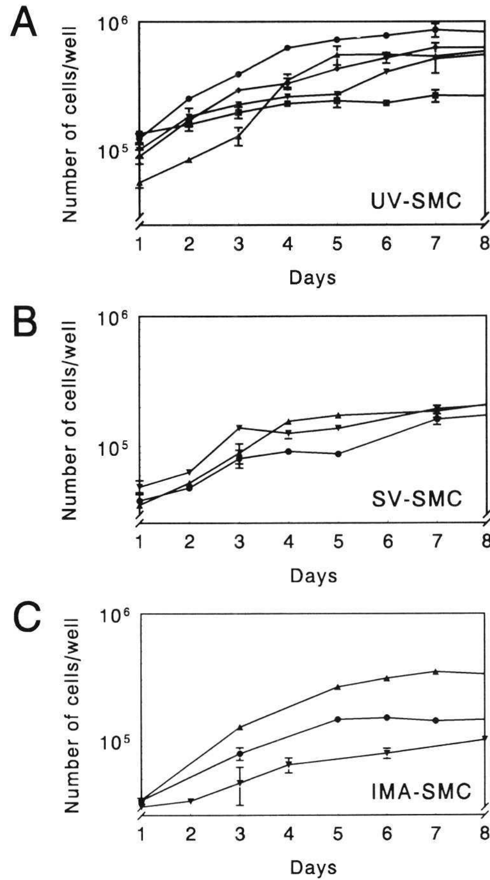

Figure 1. Growth curves of human UV-SMC, SV-SMC, and IMA-SMC
SMC were seeded in a 1:3 split ratio in 6-well plates and grown in normal culture medium. At several days after seeding cells were trypsinised and counted as described in Materials and Methods. (A) UV-SMC, (B) SV-SMC, (C) IMA-SMC. Each point represents the mean of duplicate wells (± S.D.) and the curves represent SMC isolated from different patients.

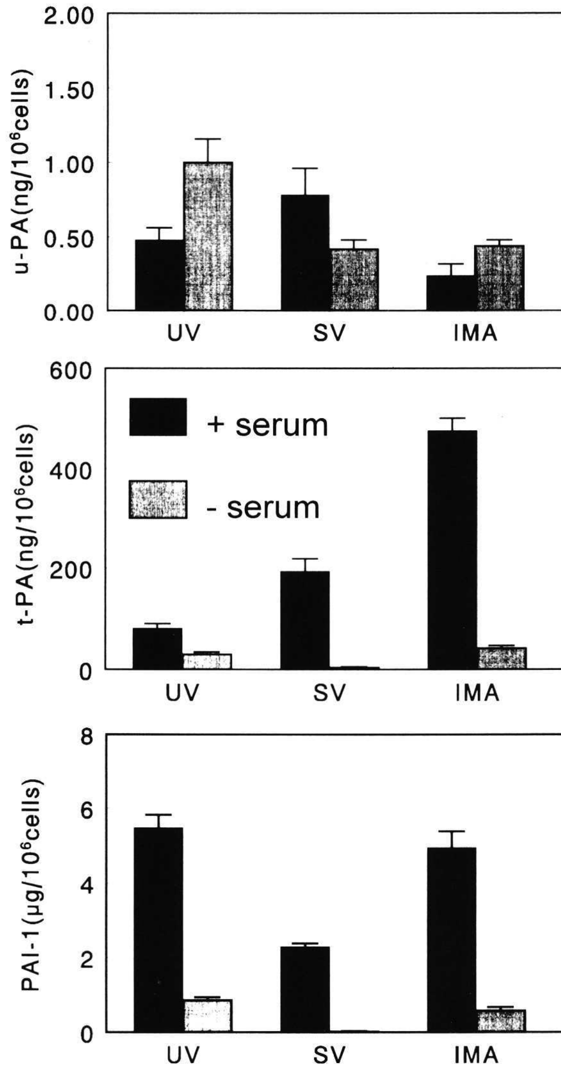

Figure 2. Concentrations of plasminogen activators and PAI-1 in the conditioned medium of human UV-SMC, SV-SMC, and IMA-SMC

Confluent UV-SMC, SV-SMC, and IMA-SMC were incubated for 24 h with 2 mL normal culture medium or 2 mL serum-free culture medium. The media were then collected and the cells were counted. In the conditioned media u-PA (A), t-PA (B), and PAI-1 (C) antigen levels were measured as described in Materials and Methods. Data represent mean ± S.E.M. (for each celltype at least four different isolations and 6 different passages were measured).

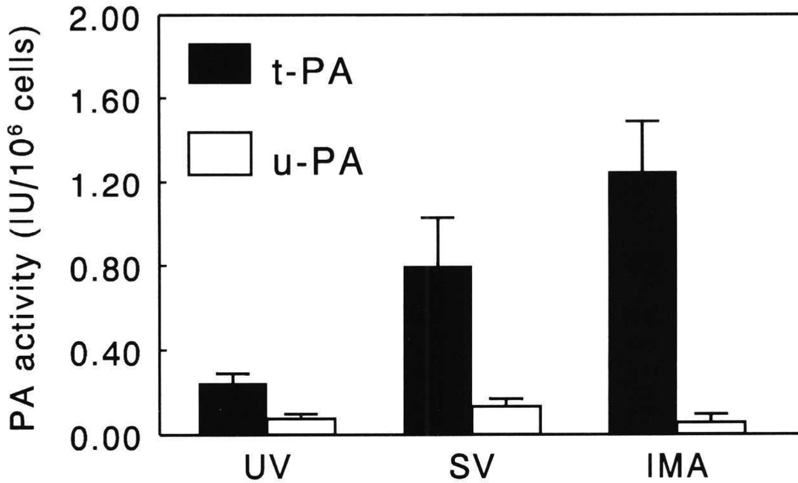

Figure 3. Plasminogen activator activity in conditioned medium of human UV-SMC, SV-SMC, and IMA-SMC

Confluent UV-SMC, SV-SMC, and IMA-SMC were incubated for 24 h with 2 mL serum-free culture medium. The medium was then collected and the cells were counted. In the medium t-PA and u-PA activity were measured as described in Materials and Methods. Data represent mean ± S.E.M (for each celltype at least two different isolations and four different passages were measured).

Expression of plasminogen activators and PAI-1 in human smooth muscle cells

Normal and serum-free 24 h-conditioned culture media of confluent human UV-SMC, SV-SMC, and IMA-SMC were assayed for the antigen levels of the plasminogen activators u-PA and t-PA, and their inhibitor PAI-1. With normal culture medium, the PAI-1 antigen levels were about the same for UV-SMC and IMA-SMC (approximately 5 $\mu g/10^6$cells), and about 2 times lower for SV-SMC (figure 2A). The t-PA antigen levels however, were 6 times higher for IMA-SMC and 2 times higher for SV-SMC compared to UV-SMC (82 $ng/10^6$cells) (figure 2B). With 24 h-conditioned serum-free culture medium, PAI-1 and t-PA antigen levels were much lower than with normal culture medium. PAI-1 levels and t-PA levels were similar for UV-SMC and IMA-SMC (approximately 0.7 $\mu g/10^6$cells for PAI-1 and 36 $ng/10^6$cells for t-PA), and much lower for SV-SMC. The antigen levels of u-PA were low (<1 $ng/10^6$cells) for all SMC-types (figure 2C), both in normal and in serum-free culture media (figure 2C).

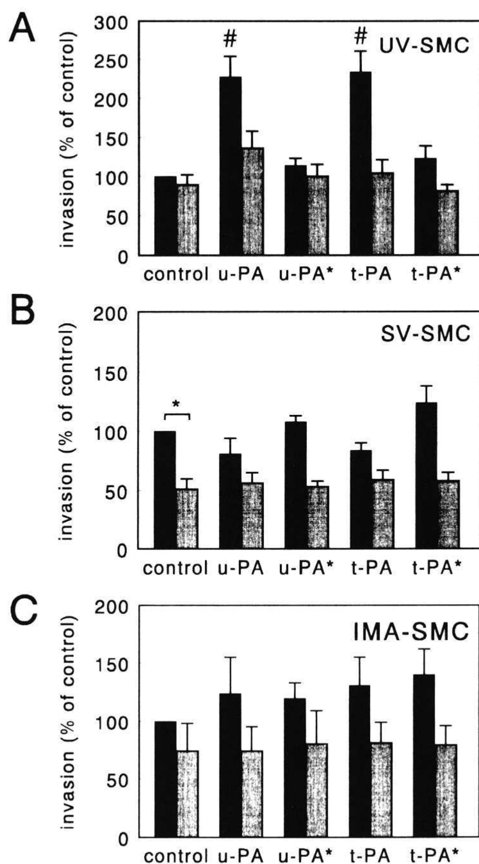

Figure 4. Invasion of human UV-SMC, SV-SMC, and IMA-SMC. Effects of aprotinin, u-PA, and t-PA

Transwell filters were coated with Matrigel. SMC suspensions (4 x 10^4 cells/filter) in serum-free culture medium with 0.15 μmol/L plasminogen were placed in the upper chamber. DMEM with penicillin (100 U/mL)/streptomycin (100 μg/mL) and 0.15 μmol/L plasminogen, containing 10% (v/v) FBS as a chemoattractant was placed in the lower chamber. To both chambers 10 nmol/L u-PA, t-PA, GGACK inactivated u-PA (u-PA*), or PPACK inactivated t-PA (t-PA*) with (hatched bars) or without (black bars) aprotinin (100 KIU/mL) were added. After several h of invasion (42-78 h for UV-SMC, 78-144 h for SV-SMC, and 66-70 h for IMA-SMC), depending on the invasion rate of the cells, invasion was measured as described in Materials and Methods and expressed as a percentage of control ± S.E.M. (no additives). (A) UV-SMC (n=5), (B) SV-SMC (n=4), and (C) IMA-SMC (n=3). # significant difference $p<0.05$ with control, * significant difference $p<0.05$.

Plasminogen activator activity

Next, in serum-free 24 h-conditioned culture medium of confluent human UV-SMC, SV-SMC, and IMA-SMC cultures, the plasminogen activator activity of t-PA and u-PA was measured (figure 3). t-PA activity was lowest in UV-SMC (0.25 IU/10^6cells), and higher in SV-SMC and IMA-SMC. u-PA activity was low in all three cell-types.

Invasion of human smooth muscle cells isolated from different vessels

The plasminogen activation system has been shown to play an important role in smooth muscle cell migration (3-7). Differences in the plasminogen activator activity between UV-SMC, SV-SMC and IMA-SMC might result in differences in the invasion capacity of these smooth muscle celltypes. We therefore investigated the role of the plasminogen activation system in the invasion of human UV-SMC, SV-SMC and IMA-SMC.

All three smooth muscle celltypes were able to invade the basement membrane matrix solution Matrigel®. We examined the role of plasmin activity, produced by the smooth muscle cell-types, in the invasion of the different smooth muscle celltypes by adding aprotinin, an inhibitor of plasmin. UV-SMC invasion (figure 4A) was not inhibited by aprotinin, while SV-SMC invasion was significantly inhibited (figure 4B). The effect of aprotinin on IMA-SMC invasion (figure 4C) showed more variability than UV-SMC and SV-SMC invasion. We measured invasion of 3 different IMA-SMC isolations. The invasion of one of those isolations was not inhibited by aprotinin, while invasion of the other two isolations was significantly inhibited (not shown).

To further determine the effect of plasminogen activation on human smooth muscle cell invasion we added HMW-u-PA or t-PA in the invasion assay. Both exogenous added u-PA and t-PA stimulated UV-SMC invasion significantly (228% and 234% of control, respectively) (figure 4A). This stimulatory effect could be inhibited by aprotinin, indicating that the stimulation of invasion by exogenous added u-PA and t-PA was caused through plasmin activity resulting from activation of plasminogen. To further substantiate this we added GGACK inactivated u-PA or PPACK inactivated t-PA. GGACK-u-PA or PPACK-t-PA did not stimulate UV-SMC invasion, implying that stimulation of UV-SMC invasion requires plasminogen activator activity. u-PA or t-PA, either active or inactive, did not stimulate SV-SMC invasion (figure 4B). IMA-SMC invasion (figure 4C) was more variable than that of UV-SMC and SV-SMC. Two isolations gave similar results as for SV-SMC and showed no stimulation whereas one isolation was stimulated by the addition of u-PA or t-PA (results not shown).

We also estimated the 'invasion rate' of smooth muscle cells by calculating the total surface area on the underside of the Transwell filters that was covered with cells in 24 h (figure 5). UV-SMC and IMA-SMC had a higher 'invasion rate' than SV-SMC. This difference was most prominent with the addition of aprotinin, u-PA or t-PA in the invasion assay.

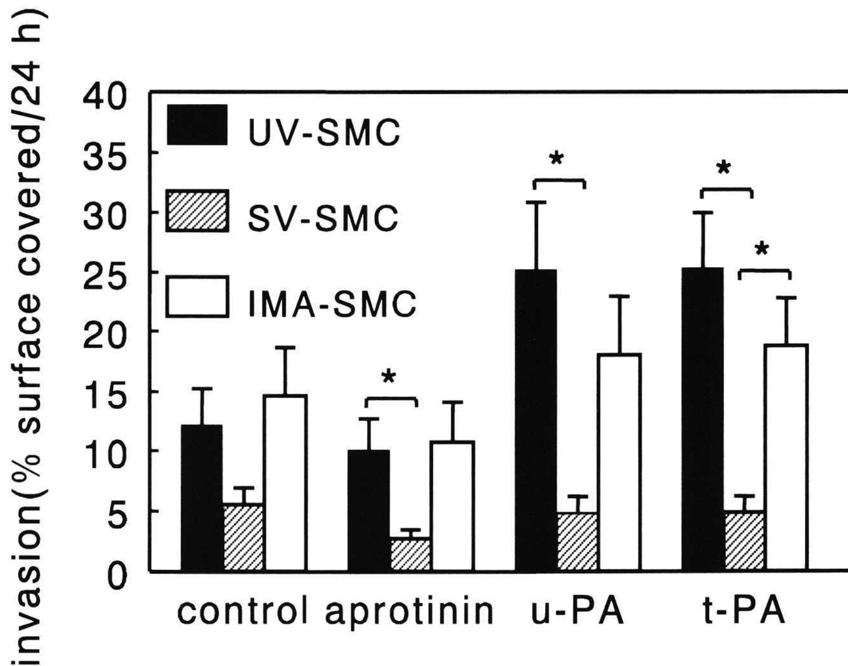

Figure 5. Invasion of human UV-SMC, SV-SMC, and IMA-SMC. Percentage of the area covered per 24 h with control, aprotinin, u-PA or t-PA

Transwell filters were coated with Matrigel. SMC suspensions (4 x 10^4 cells/filter) in serum-free culture medium with 0.15 μmol/L plasminogen were placed in the upper chamber. DMEM with penicillin (100 U/mL)/streptomycin (100 μg/mL) and 0.15 μmol/L plasminogen, containing 10% (v/v) FBS as a chemoattractant was placed in the lower chamber. To both chambers nothing (control), 100 KIU/mL aprotinin, 10 nmol/L u-PA or 10 nmol/L t-PA was added. After several h of invasion (42-78 h for UV-SMC, 78-144 h for SV-SMC, and 66-70 h for IMA-SMC), depending on the invasion rate of the cells, invasion was measured as described in Materials and Methods. The percentage of the area covered by the cells per 24 h was calculated and is expressed as mean±S.E.M. (UV-SMC (n=5), SV-SMC (n=4), and IMA-SMC (n=3)). * significant difference $p<0.05$.

Discussion

It has been established that vascular intima smooth muscle cells are phenotypically distinct from vascular media smooth muscle cells (19-21). Different phenotypic smooth muscle cells could also be present in distinct human blood vessels (11), and may determine the heterogeneity of vessels to develop vascular diseases like atherosclerosis and restenosis. Proliferation and migration are important processes in the formation of (neo)intimal thickening during the atherosclerotic process and in restenosis (22). In this study we therefore isolated smooth muscle cells from different types of vessel (internal mammary artery, saphenous vein, and umbilical vein), and compared their growth and migration properties.

We did not notice any significant differences in the growth rates between SV-SMC, IMA-SMC, and UV-SMC. However, in the conditioned medium of both serum-stimulated and serum-depleted smooth muscle cellcultures, we found clear differences in the concentrations of t-PA, u-PA and PAI-1 between UV-SMC, SV-SMC, and IMA-SMC. This resulted in a difference in plasminogen activator activity in the medium of the smooth muscle celltypes. While plasminogen activation activity was similar for SV-SMC and IMA-SMC, it was much lower for UV-SMC.

In previous studies we showed that the plasminogen activation system is involved in human vascular smooth muscle cell invasion *in vitro* (6,7). Other investigators have also found an important role for the plasminogen activation system in smooth muscle cell migration *in vivo* (3-5). Differences in the plasminogen activator activity between UV-SMC, SV-SMC, and IMA-SMC might result in differences in the invasion capacity between the smooth muscle celltypes. Indeed, we found a correlation between plasminogen activator activity and the invasion properties of the smooth muscle celltypes. Invasion of the smooth muscle celltype with the lowest plasminogen activator activity (UV-SMC) was not inhibited by the addition of aprotinin, an inhibitor of plasmin activity. In contrast, invasion of smooth muscle celltypes with the highest plasminogen activator activity (SV-SMC and IMA-SMC) was inhibited by aprotinin. This suggests that in UV-SMC not enough plasmin activity can be generated to induce plasmin-mediated invasion, while in SV-SMC and in IMA-SMC enough plasmin activity can be generated to mediate invasion. In support of this, UV-SMC invasion was stimulated by the addition of u-PA or t-PA, while SV-SMC and IMA-SMC invasion were not. These results show that the balance between plasminogen activators and inhibitors can have great influence on the invasion capacity of cells and that smooth muscle cells regulate their own migration capacity in part by the regulation of their plasminogen activator activity.

It is known that the plasminogen activation system can not only exert an effect on cell migration via the proteolytic breakdown of the extracellular matrix surrounding cells, but also via other mechanisms. PAI-1 has been shown to be an important regulator of smooth muscle cell migration, because it can inhibit smooth muscle cell adhesion by blocking the binding of the vitronectin receptor to the extracellular matrix protein vitronectin (23). The cellular receptor for u-PA, u-PAR, can stimulate the adhesion of cells via its binding to vitronectin (24,25). Binding of u-PA to u-PAR can, independently of its proteolytic activity, induce intracellular signalling pathways involved in migration and proliferation (26,27). In our study however, we did not notice any non-proteolytic effects of t-PA or u-PA. The addition of inactivated u-PA or t-PA did not have any stimulatory effect on smooth muscle cell invasion in the three smooth muscle celltypes. Furthermore, the stimulatory effects of u-PA or t-PA in UV-SMC could also be completely inhibited by the addition of the plasmin inhibitor aprotinin. We can however not rule out the possibility that PAI-1 affects smooth muscle cell invasion via its binding to vitronectin.

In our invasion model we could only inhibit smooth muscle cell invasion through Matrigel, in part (SV-SMC) or not at all (UV-SMC) with aprotinin. SV-SMC invasion could only be inhibited by aprotinin for maximal 50%. This suggests that other proteases, like for example matrix metalloproteinases may also be involved in smooth muscle cell invasion (1,2).

In our human *in vitro* system both t-PA and u-PA appear to be involved in smooth muscle cell migration/invasion both via plasmin formation. These results differ from the observations of Carmeliet and co-workers who found a dependence of u-PA and not t-PA on *in vivo* smooth muscle migration/invasion in the mouse (28-32). At present we do not know whether this difference is due to the different species (human as compared with mouse) or different experimental design (*in vitro* as compared with *in vivo*).

Interestingly, Gabbiani *et al.* also found a correlation between smooth muscle cell phenotype, migration and plasminogen activator expression (20,21). They isolated two phenotypic distinct smooth muscle cell variants from rat aorta: a spindle-shaped phenotype, obtained from normal adult aorta, and an epitheloid phenotype, obtained from intimal thickening. Epitheloid smooth muscle cells were able to grow without serum and migrated faster than spindle smooth muscle cells. Epitheloid smooth muscle cells also displayed a higher plasminogen activator activity than spindle-shaped smooth muscle cells. This was due to differences in the expression of t-PA, to a lesser extent of u-PA, and was accompanied by a decrease in PAI-1 in epitheloid cells as compared to spindle-shaped smooth muscle cells.

In contrast to Gabbiani we did not study the morphology of the smooth muscle cell-types in detail. We however did notice that there were differences in the amount of cells/cm^2 in confluent smooth muscle cell cultures. Confluent (95-100% of surface area covered with cells) UV-SMC layers had more cells/cm^2 than SV-SMC and IMA-SMC layers, indicating that UV-SMC are smaller than IMA-SMC and SV-SMC. When we estimated the 'invasion rate' by calculating the total surface area on the underside of the Transwell filters that was occupied with cells after 24 hours, we noticed that UV-SMC and IMA-SMC migrated 'faster' than SV-SMC. When we take into account that UV-SMC are probably smaller than the other two smooth muscle celltypes, we have even underestimated the 'real' UV-SMC invasion rate (the *number* of cells that migrate through the filter/24 hours). This indicates that besides differences in plasminogen activator and PAI-1 expression, there are also other phenotypical differences between the smooth muscle celltypes that can influence invasion. For example, other proteases such as the metalloproteinases might be differently expressed between the smooth muscle celltypes (1,2).

In conclusion, in this study we were able to show that there are differences in the invasion capacity of smooth muscle cells isolated from different human vessels. In part the differences in invasion could be explained by a difference in the expression of plasminogen activators and PAI-1 between smooth muscle cells isolated from different vessels. Our results suggest that phenotypic differences exist between human vessel smooth muscle cells, and might explain the variability of vessels to develop vascular disease.

Acknowledgments

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Chapter 5

Regulation of tissue-type plasminogen activator and PAI-1 accumulation by cell-density in human vascular smooth muscle cells. Role for members of the LDL-receptor family

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Submitted

Summary

It has been demonstrated that the plasminogen activation system plays a role in the migration of human vascular smooth muscle cells (HVSMC) in vitro. For an efficient migration to occur it is necessary that the amount and activity of plasminogen activators are tightly regulated. In this study, we therefore determined the generation of tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), and their main physiological inhibitor PAI-1 by HVSMC. After 24 h of culture, the levels of t-PA and PAI-1 ceased to increase in conditioned medium, while u-PA levels continued to rise in time. There was a strong negative correlation between cell-density and the levels of t-PA and PAI-1 measured per cell in 24 h conditioned medium, while u-PA levels were not correlated with cell-density. These observations could not be explained by differences in gene expression, since t-PA and PAI-1 mRNA levels were higher in high, compared to low cell-density cultures. We investigated therefore, whether these results could be explained by a difference in the binding and uptake of t-PA and PAI-1 between high and low-density cultures. HVSMC are known to express two members of the LDL-receptor family, LDL-receptor related protein (LRP) and the VLDL-receptor. Both receptors are involved in the binding and clearance of t-PA and t-PA:PAI-1 complexes. t-PA binding and association could only in high cell-density cultures be efficiently inhibited by RAP (receptor associated protein), a protein which inhibits the binding of LRP and the VLDL-receptor. When RAP was added to high or low-density cultures, we observed an increase in the amount of t-PA per cell accumulating in the medium only in high cell-density HVSMC. There was no difference in LRP protein level between high and low density HVSMC, but VLDL-receptor mRNA levels were higher in high compared to low cell-density HVSMC. These results suggest that the cell-density dependent accumulation of t-PA and PAI-1 is regulated by the uptake of t-PA and PAI-1 via members of the LDL-receptor family, most probably via the VLDL-receptor.

Introduction

Migration and proliferation of smooth muscle cells (SMC) are important processes in the development of arteriosclerosis and the pathogenesis of restenosis. One of the factors that play a role in these processes is the controlled degradation of the extracellular matrix (ECM) that surrounds and supports SMC. Among the proteolytic enzymes that are known to be involved in ECM degradation is plasmin. Next to its ability to degrade fibrin, plasmin can degrade a broad range of ECM molecules, like laminin, fibronectin, vitronectin and proteoglycans. It can also activate latent metalloproteinases or growth factors. Plasmin is formed from the inactive

proenzyme plasminogen by tissue-type (t-PA) or urokinase-type (u-PA) plasminogen activators. The main physiological inhibitor for these plasminogen activators is plasminogen activator inhibitor type-1 (PAI-1).

In animal vascular injury models, a functional link between plasminogen activation and SMC migration has been observed. In a rat carotid balloon injury model, Clowes et al. (1) observed an induction of both u-PA and t-PA expression, with u-PA expression correlating with the time of SMC proliferation and t-PA expression correlating with the time of migration of SMC from the media to the neointima. In transgenic mice with targeted disruption of the u-PA or plasminogen gene, intimal thickening in response to carotid injury is reduced, whereas in mice with disruption of the PAI-1 gene, intimal thickening is increased (2,3). Mice with disruption of the t-PA gene have, however, a similar intimal thickening as control mice. In a previous article we have demonstrated that the plasminogen activation system is also involved in human vascular SMC migration in vitro (4,5).

The ability of SMC to regulate the activity of plasminogen activators is an important aspect in the response of these cells to vessel wall injury. For an efficient migration to occur it is necessary that the activity of plasminogen activators is tightly regulated. In this study we therefore analysed the generation of plasminogen activators and their main physiological inhibitor PAI-1 by cultured human vascular SMC (HVSMC). We demonstrate in this study that the accumulation in the culture medium of t-PA and PAI-1 by SMC is negatively correlated with cell-density, while there is no such effect of cell-density on t-PA or PAI-1 mRNA expression. Our results suggest that the dependency of the accumulation of t-PA and PAI-1 on cell-density is regulated at least in part by the uptake of t-PA and/or t-PA:PAI-1 complexes via members of the LDL-receptor family, most probably via the VLDL-receptor.

Materials and Methods

Cell culture

Human vascular smooth muscle cells (HVSMC) were isolated from unused pieces of saphenous vein which were obtained after by-pass surgery. After gentle removal of endothelial cells, the adventitia was carefully stripped from the media. The media was then cut into small fragments. SMC either were isolated via the explant method (6), or by incubating media fragments overnight with 0.075% (w/v) collagenase (Worthington, Biochemical Corp. Freehold, NJ, USA) in culture medium [Dulbecco´s modified Eagle medium (DMEM, Gibco BRL, Paisley, Scotland), supplemented with 5% (v/v) heat inactivated (30 min, 56 °C) foetal bovine serum (FBS, Life Technologies, Paisley, Scotland), 5% (v/v) heat inactivated human serum (local blood bank) and streptomycin (100 μg/mL)/penicillin (100 U/mL) (Biowhittaker, Verviers, Belgium)].

HVSMC were cultured in culture medium on gelatine (Merck, Darmstadt, Germany) coated dishes at 37°C in a humidified 5% CO_2/95% air (v/v) atmosphere. The medium was replaced every 3 days and subcultures were established by trypsin/EDTA treatment of confluent cultures at a split ratio of 1:3. HVSMC were used for experiments between passage 2-6.

Enzyme-linked immunosorbent assays
Assay of u-PA antigen was performed as described (7). t-PA antigen was determined with the Thrombonostika t-PA ELISA (Organon Teknika, Boxtel, The Netherlands), and PAI-1 antigen with IMULYSE^{TM} PAI-1 (Biopool, Umeå, Sweden).

mRNA analysis
HVSMC were washed with phosphate buffered saline (PBS) and lysed in 4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.5, 5 g/L Sarkosyl, 0.1 M 2-mercaptoethanol. RNA was isolated according to Chomczynski and Sacchi (8). RNA samples were electrophoresed on a 1.2% (w/v) denaturing agarose gel containing 7.5% (v/v) formaldehyde and were transferred to Hybond N membrane (Amersham, Buckinghamshire, United Kingdom) using a Vacugene system (Pharmacia, Sweden). Membranes were hybridised with ^{32}P-labelled cDNA fragments in 7% (w/v) sodium dodecyl sulphate (SDS), 0.5 M sodium phosphate pH 7.2, 1 mM EDTA at 62°C. Blots were washed with SSC (0.15 M NaCl, 0.15 M sodium citrate), 1% (w/v) SDS at 62°C and analysed using a phosphorimager (Fuji Bas1000, Raytest).

cDNA probes
cDNA fragments were labelled using the random primer method (Megaprime, Amersham, United Kingdom), with ^{32}P-dCTP. The following cDNA fragments were used as probes: a 1.9 kb *Bgl II* fragment of the human t-PA cDNA, a 1.1 kb *Pst I* fragment of the human PAI-1 cDNA, a 752 kb fragment of the human VLDL-receptor cDNA, kindly provided by Dr. K. Willems van Dijk and a 1.2 kb *Pst I* fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

Labelling of t-PA and RAP protein
t-PA (Actilyse, Boehringer Ingelheim, Germany) and RAP [produced in E. coli strain DH5α as a fusion protein with glutathione S-transferase (GST-RAP) and purified by glutathione-Sepharose chromatography as described (9)] were labelled with Na^{125}I using the iodogen method (10). Polyethylene vessels of 1.5 mL were coated with 10 µg iodogen. Subsequently, 10 µg t-PA or 7.5 µg RAP was added in 0.1 M Tris pH 7.6 + 0.01% (v/v) Tween-80 and incubated with 10 µL Na^{125}I (activity 100 µCi/µL) for 1 h at room temperature. Labelled t-PA was separated from free ^{125}I using a Zinc-Sepharose column (elution buffer 0.02 M Tris pH 7.5, 1 M NaCl, 0.05 M Imidazol, 0.01% (v/v) Tween-80) and a Sephadex G-50 fine column. GST-RAP was separated from free label with a Sephadex G-25 column.

Ligand blot
HVSMC were grown to high and low cell-density and incubated for 24 h in fresh culture medium. Cells were washed with PBS and detached with 0.05% (w/v) EDTA. After washing the cells with PBS, the cell-pellet was lysed in 0.1% (v/v) Triton X-100 in PBS and sonicated. Thereafter, lysates were centrifuged (10 min, 1000g). In the supernatant the amount of protein was determined with the

BCA protein assay (Pierce, Rockford, Illinois, USA). 50 µg of protein was subjected to gel-electrophoresis on a 5% SDS-PAGE gel, and thereafter electrophoretically transferred to a nitro-cellulose filter (Schleicher and Schuell, Dassel, Germany) using a semi-dryblott apparatus (Pharmacia). The filter was blocked in washing-buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween-20) with 1% (w/v) non fat dry milk (NFDM), and thereafter washed with washing buffer with 0.5% (w/v) NFDM. The blot was incubated for 3 h in 5 mL washing-buffer with 0.5% (w/v) NFDM and 1 µg ^{125}I-GST-RAP. Thereafter the filter was very extensively washed with washing-buffer. Bound GST-RAP was quantified with a phosphorimager (Fuji Bas1000, Raytest).

Binding, association and degradation of ^{125}I -tPA by high and low cell-density HVSMC cultures
For binding, association and degradation studies, HVSMC were grown to high and low cell-density in 24-well plates. They were washed twice with 0.5 mL PBS, 1% (w/v) bovine serum albumin (BSA) at 4°C. ^{125}I-t-PA with or without inhibitors (unlabelled t-PA or GST-RAP) was added in 0.4 mL DMEM, 1% (w/v) BSA, 0.01% (v/v) Tween-80.
For binding studies, cells were incubated for 2 h at 4°C. Thereafter the medium was removed and the cells were quickly washed five times with PBS, 1% (v/v) BSA at 4°C. The cells were then lysed in 0.3 mL 0.2 M NaOH for at least 15 minutes at 37°C. Radioactivity of the lysates was determined in a γ-counter. Non-specific binding of ^{125}I-t-PA to plastic or serum-components was determined in parallel wells not containing cells.
For association and degradation studies, cells were incubated for 4 h at 37°C. After incubation, the cells were washed and lysed as described for binding. The amount of radioactivity in the lysates represents the associated amount of ^{125}I-t-PA. The amount of degraded ^{125}I-t-PA was determined in the cell-media. TCA (final concentration 10% (w/v)) was added to the cell-media and non-degraded ^{125}I-t-PA was precipitated by centrifugation (10 min at 15.000g). Remaining free ^{125}I was removed from 150 µL of the TCA soluble supernatant by adding 1.5 µL KI (40% w/v) and 7.5 µL H_2O_2 (30% v/v). After 5 min, the free iodine was extracted with 240 µL chloroform. After centrifugation (5 min, 15.000g), the radioactivity in the upper layer was counted.

Results

Influence of cell-density on the accumulation of plasminogen activators and PAI-1 by human vascular smooth muscle cells in culture

Culture media of human vascular smooth muscle cells (HVSMC), isolated from saphenous vein, were assayed for the accumulation of the plasminogen activators u-PA and t-PA, and their inhibitor PAI-1 in time. We found that u-PA levels rose in time, with a maximal level of 13 ng/10^6 cells measured after 120 h (figure 1A). t-PA levels, however, reached a maximal level at 24 h (230 ng/10^6 cells) and thereafter steadily declined (figure 1B). HVSMC produced large amounts of PAI-1 (3.4 µg/10^6 cells after 24 h) which ceased to accumulate after 24 h. Only at 120 h there was an additional increase in PAI-1 levels observed (figure 1C).

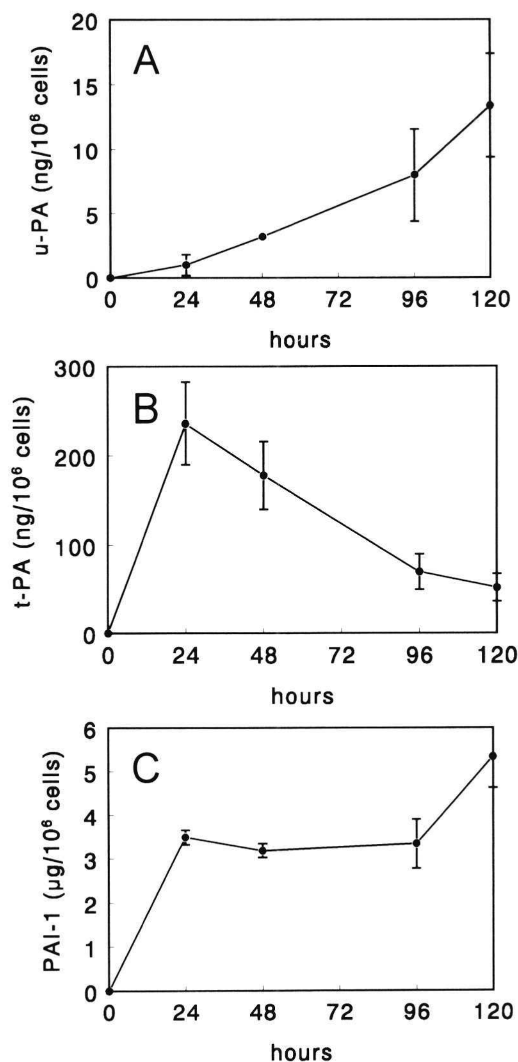

Figure 1. Concentrations of t-PA, u-PA and PAI-1 in conditioned media of HVSMC
Confluent HVSMC in 10 cm^2 wells were incubated for various time periods with 2 mL of culture medium. At each time point indicated, media were collected and cells were counted. Analyses of u-PA (A), t-PA (B) and PAI-1 (C) antigen levels in conditioned media were performed as described in Materials and Methods. Each point represents the mean ± SD of triplicate wells.

The reduction in t-PA levels observed after 24 h could be caused by the degradation of t-PA in the medium. To test this, we spiked 48 h conditioned medium of HVSMC (t-PA concentration 7.2 ng/mL) with 30 ng/mL t-PA and incubated this for several h at 37°C (figure 2). Even after 7 days, we found no significant decrease in the amount of t-PA measured.

In 24 h conditioned medium, we noticed an inverse correlation between the level of t-PA/cell and the number of cells/cm² (figure 3A). A similar correlation was found for PAI-1 (figure 3B), but for u-PA there was no correlation between the u-PA level/cell and cell-density (figure 3C). Similar results were obtained for SMC isolated from umbilical vein or left internal mammary artery (results not shown).

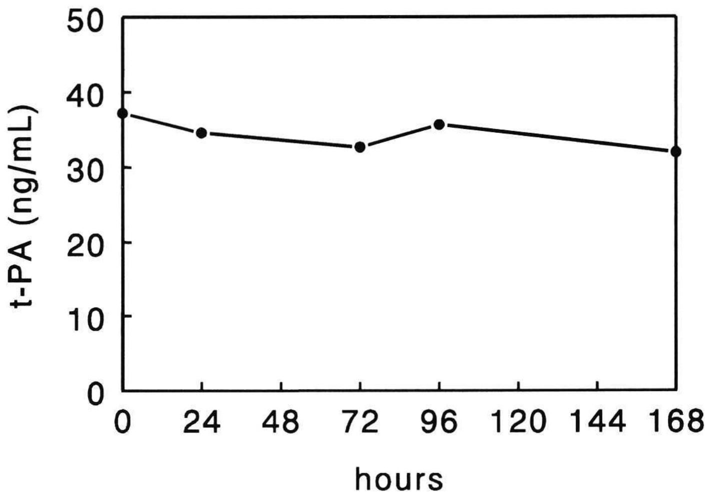

Figure 2. Incubation of t-PA in conditioned medium of HVSMC
30 ng/mL t-PA was added to 48 h conditioned medium of HVSMC (t-PA concentration 7.2 ng/mL) and incubated for several h at at 37°C. At each time point indicated, media was collected, and analyses of t-PA antigen levels were performed as described in Materials and Methods. Each point represents the mean of duplicate measurements.

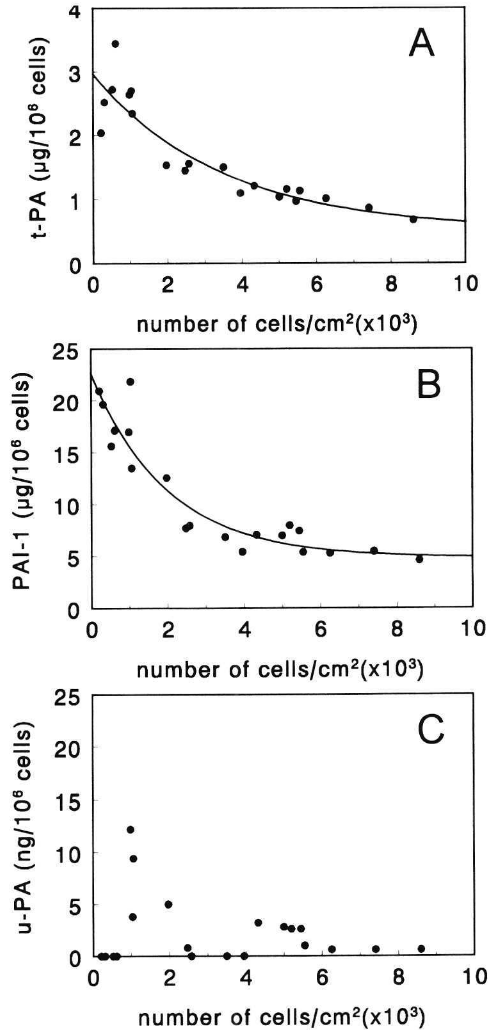

Figure 3. Effect of cell-density on the concentrations of t-PA, PAI-1 and u-PA in the conditioned medium of HVSMC

HVMC were seeded at different cell-densities in 10 cm^2 wells and grown for 3-4 days. Fresh culture medium was then added to the cells and incubated for 24 h. Media were collected and the cells were counted with a Bürker-Türk chamber. Analyses of t-PA (A), PAI-1 (B), and u-PA (C) antigen levels in conditioned media were performed as described in Materials and Methods. Each point represents the mean of duplicate wells. Curves were fitted by using the curve-fit algorithm of SlideWrite Plus (Advanced Graphics software, Inc. Carlsbad, USA).

mRNA expression of t-PA and PAI-1 in low and high cell-density HVSMC

The observed differences in t-PA and PAI-1 accumulation between low and high cell-densities could be caused by several mechanisms. Regulation could be at the level of gene-expression, protein synthesis, protein secretion, or via protein uptake and degradation by the cell. We first examined if there were differences in the expression of t-PA and/or PAI-1 mRNA in high and low cell-density HVSMC.

In three separate experiments, Northern blot analysis showed an *increase* in t-PA mRNA levels (198%) and an *increase* in PAI-1 mRNA levels (147%), relative to GAPDH, in high cell-density cultures as compared to low cell-density cultures (figure 4). The expression of mRNA can therefore not be an explanation for the negative correlation between cell-density and t-PA or PAI-1 levels.

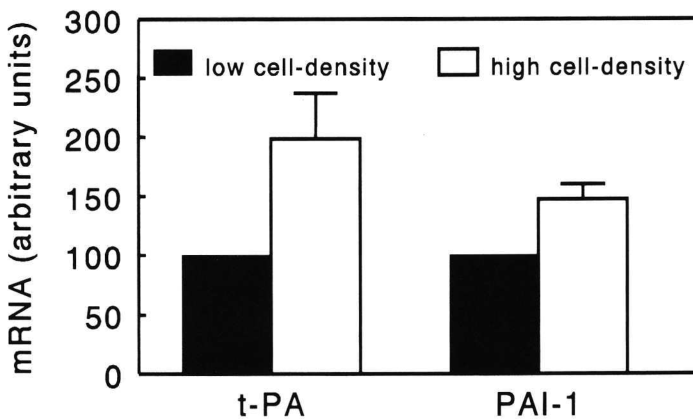

Figure 4. Northern blot analysis of HVSMC t-PA and PAI-1 mRNA at different cell-densities
Total RNA was isolated from low and high cell-density HVSMC cultures. 10 μg RNA was subjected to gel electophoresis and transferred to nylon membranes. Blots were hybridised with t-PA and PAI-1 cDNA probes. The amounts of t-PA and PAI-1 mRNA were quantified with a phosphorimager and corrected for uneven RNA loading with GAPDH as an internal standard.

Binding, uptake and degradation of ^{125}I-t-PA by high and low cell-density HVSMC. Involvement of members of the LDL-receptor family.

Because the results of figure 1 suggested that part of the endogenous produced t-PA is taken up by HVSMC we studied if there were differences in the binding, uptake and degradation of t-PA between high and low cell-density cultures. Displacement studies of 1 nM ^{125}I-t-PA with

unlabelled t-PA showed no differences in the binding-curves for high and low-density cultures (figure 5). Similar K_i values (0.8 nM) were obtained for high and low cell-density cultures. When the number of binding sites/cell for t-PA were estimated, low-density HVSMC had however approximately 7 fold more binding-sites/cell than high-density HVSMC.

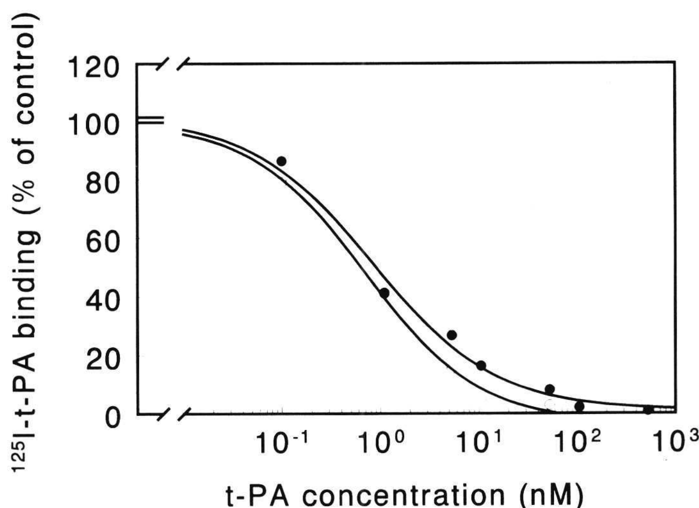

Figure 5. Binding of t-PA in high and low cell-density HVSMC
HVSMC at a high (●) and low (○) cell-density were incubated with 1 nM ^{125}I-t-PA and the indicated concentrations of unlabelled t-PA for 2 h at 4°C. Thereafter cells were washed thoroughly and lysed. In cell-lysates radioactivity was measured as described in Materials and Methods. Data are expressed as percentage of specific control binding and points represent the mean of triplicate wells. 100 % binding was for low-density cultures $9x10^5$ cpm/10^6cells and for high-density cultures $1.3x10^5$ cpm/10^6cells. Aspecific binding to plastic was 16% of total binding. Curves were fitted by using the DoseRspLgstc curve-fit algorithm of SlideWrite Plus (Advanced Graphics software, Inc. Carlsbad, USA).

It has been shown that two members of the LDL-receptor family, LRP and the VLDL-receptor, are involved in the binding and uptake of a number of ligands, including pro-u-PA, t-PA and plasminogen activator:PAI-1 complexes (11-13). In SMC, several investigators (14,15) have demonstrated these receptors. We examined if these receptors were involved in the binding of t-PA on low and high cell-density HVSMC with GST-RAP, which is an antagonist for the binding to LRP and the VLDL-receptor (16,17). In low density-HVSMC unlabelled GST-RAP could not displace the specific binding of ^{125}I-t-PA. In high density

cultures however, GST-RAP could displace about 60% of the specific binding of ^{125}I-t-PA, with an apparent K_i for binding of 0.3 nM (figure 6).

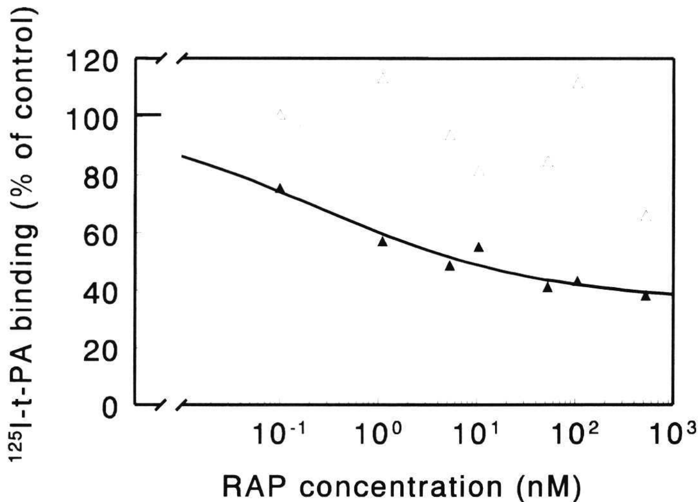

Figure 6. Binding of t-PA in high and low cell-density HVSMC. Effect of GST-RAP
HVSMC at a high (35.4×10^3 cells/cm^2) (▲) and low (5.5×10^3 cells/cm^2) (△) cell-density were incubated with 1 nM ^{125}I-t-PA and the indicated concentrations of unlabelled GST-RAP for 2 h at 4°C. Thereafter cells were washed thoroughly and lysed. In cell-lysates radioactivity was measured as described in Materials and Methods. Data are expressed as percentage of specific control binding and points represent the mean of duplicate wells. 100% binding was for low-density cultures 9.2×10^5 cpm/10^6cells and for high-density cultures 1.3×10^5 cpm/10^6cells. Aspecific binding to plastic was 16% of total binding. Curves were fitted by using the DoseRspLgstc curve-fit algorithm of SlideWrite Plus (Advanced Graphics software, Inc. Carlsbad, USA).

We next investigated if there were differences in the association and degradation of ^{125}I-t-PA between high and low cell-density cultures. In high cell-density HVSMC the association (figure 7A) and degradation (figure 7B) of ^{125}I-t-PA were completely blocked by unlabelled t-PA as well as by GST-RAP. Association in low cell-density HVSMC could be completely blocked by unlabelled t-PA, and could be inhibited for 40% by GST-RAP (figure 8). Unfortunately, the degradation of ^{125}I-t-PA could not be accurately determined in low cell-density cultures, due to very low radioactive counts compared to background levels.

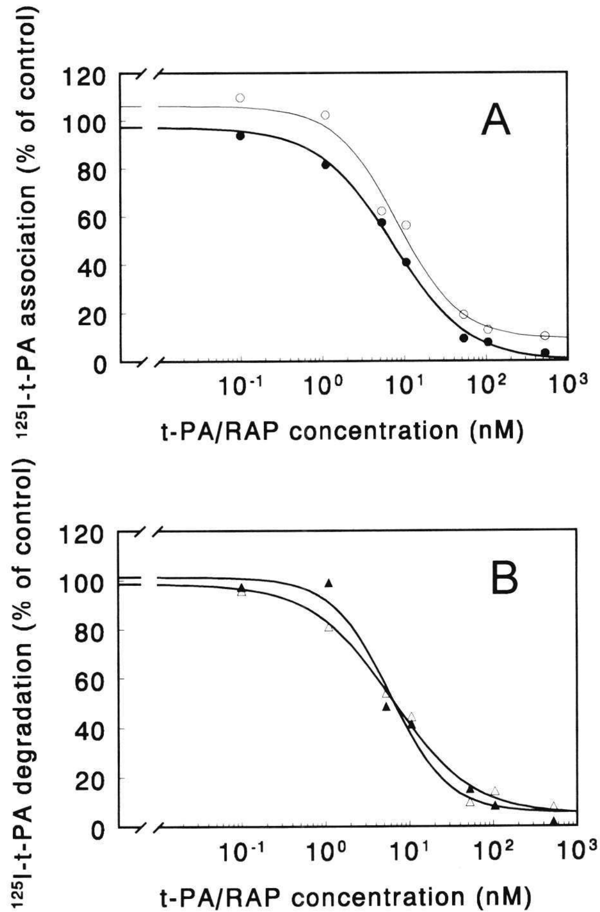

Figure 7. Association and degradation of t-PA in high cell-density HVSMC. Effect of GST-RAP HVSMC at a high cell-density ($35.4x10^3$ cells/cm^2) were incubated with 1 nM ^{125}I-t-PA and the indicated concentrations of unlabelled t-PA (▲●) or GST-RAP (△○) for 4 h at 37°C. Thereafter degradation was measured in medium and association was measured in cell-lysates as described in Materials and Methods. Data are expressed as percentage of specific control for association (A) or degradation (B). Points represent the mean of duplicate or triplicate wells. 100 % was for association $9.3x10^5$ cpm/10^6cells and for degradation $15.3x10^5$ cpm/10^6cells. Aspecific binding to plastic was 16% of total binding. Curves were fitted by using the DoseRspLgstc curve-fit algorithm of SlideWrite Plus (Advanced Graphics software, Inc. Carlsbad, USA).

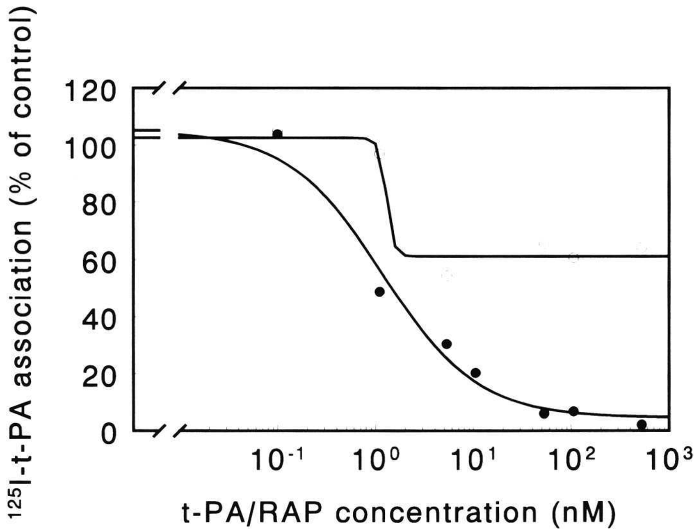

Figure 8. Association of t-PA in low cell-density HVSMC. Effect of GST-RAP
HVSMC at a low cell-density ($5.5x10^3$ cells/cm^2) were incubated with 1 nM ^{125}I-t-PA and the indicated concentrations of unlabelled t-PA (●) or GST-RAP (○) for 4 h at 37°C. Thereafter association was measured in cell-lysates as described in materials and methods. Data are expressed as percentage of specific control for association. Points represent the mean of duplicate or triplicate wells. 100 % was for association $15.5x10^5$ cpm/10^6cells. Aspecific binding to plastic was 16% of total binding. Curves were fitted by using the DoseRspLgstc curve-fit algorithm of SlideWrite Plus (Advanced Graphics software, Inc. Carlsbad, USA).

Accumulation of t-PA in high and low cell-density HVSMC cultures in the presence of GST-RAP

Because the results of figures 6-8 suggested that members of the LDL-receptor family may play a role in the cell-density dependent accumulation of t-PA, we measured t-PA antigen levels in 24 h conditioned cell-medium at high and low cell-density in the presence or absence of 10 µg/ml GST-RAP. The addition of GST-RAP to HVSMC had no effect on the level of t-PA in the medium of low cell-density cultures, whereas there was an 80% increase in t-PA levels in high cell-density cultures (figure 9).

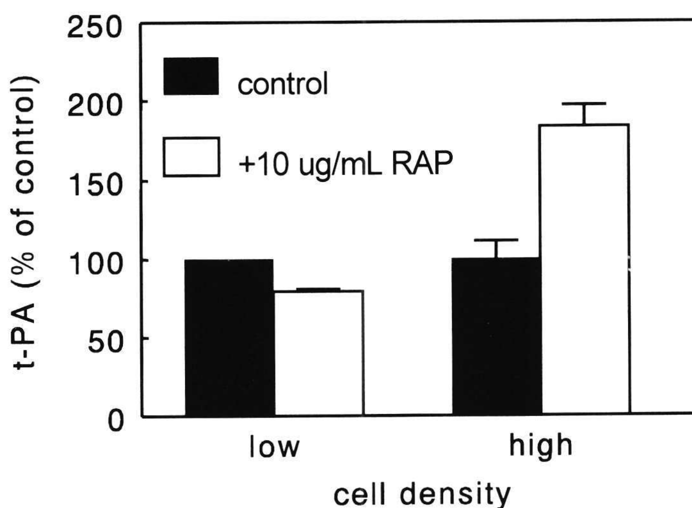

Figure 9. Effect of RAP on the accumulation of t-PA by low and high cell-density HVSMC cultures

High density HVSMC cultures ($29x10^3$ cells/cm^2) and low density HVSMC cultures ($2.9x10^3$ cells/cm^2) were incubated for 24 h in 2 mL culture medium with or without (control) 10 µg/mL RAP-GST. Media were collected and the cells were counted. Analysis of t-PA antigen levels in the media was performed as described in Materials and Methods. t-PA levels are expressed as percentage of control (i.e. without GST-RAP added). Control values are 1.1 µg/well for low density cultures and 0.2 µg/well for high density cultures. Data are expressed as mean ± SD of duplicate wells.

Analysis of the expression of members of the LDL-receptor family in high and low cell-density HVSMC

Finally, we examined if there were differences in the expression of LRP and/or the VLDL-receptor between high and low cell-density cultures. Ligand blot analysis was performed on cell-lysates of high and low cell-density HVSMC, with ^{125}I-GST-RAP. Bound GST-RAP was quantified with phosphor-imager analysis. LRP protein was detected in both high and low cell-density cultures (figure 10), but there was no significant difference between low (100%) and high cell-density (132 ± 63 %, n=2). VLDL-receptor could not be detected with the ligand blot method, but on Northern blot, VLDL-receptor mRNA was increased with 294 ± 33 % (n=2) in high cell-density cultures compared to low cell-density cultures.

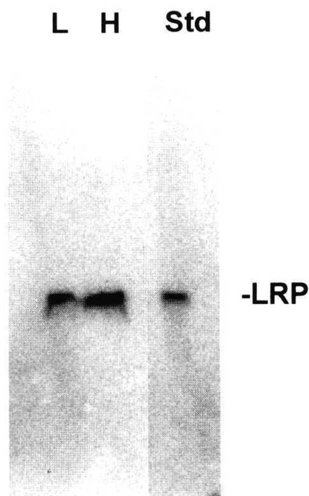

Figure 10. ^{125}I-GST-RAP ligand blot analysis in high and low cell-density HVSMC
50 μg of cell-lysate from high (H) and low (L) cell-density HVSMC were subjected to gel electrophoresis and thereafter transferred to nitro-cellulose filters. As a standard (Std) a mouse liver extract was used, which is known to contain LRP. Ligand blot was performed with ^{125}I-GST-RAP as described in Materials and Methods.

Discussion

After vascular injury SMC start to proliferate and migrate from the media to the (neo)intima, processes which are necessary to repair the damaged vascular wall. For migration and matrix remodelling processes, the proteolytic degradation of the extracellular matrix that encages cells is essential. Several investigators have shown that the plasminogen activation system is involved in the migration and invasion of SMC (1-5). For an efficient migration to occur it is necessary that the plasminogen activator activity is tightly regulated. In this study we therefore investigated the generation of t-PA, u-PA and their inhibitor PAI-1 by human vascular SMC. We show that there are differences in the accumulation of plasminogen activators and PAI-1 in the medium of HVSMC in time. While the u-PA level continuously rises in time, PAI-1 ceases to accumulate after 24 h. t-PA reaches a maximum level after 24 h, and thereafter steadily declines. Because the reduction of t-PA antigen level after 24 h was not found to be due to protein instability or proteolytic breakdown in the culture medium this suggests that the available amounts of endogenous produced t-PA are at least in part regulated via uptake and degradation by HVSMC.

While the accumulation of u-PA in the culture medium did not change with increasing cell-density, there was a strong negative correlation between the amounts of t-PA and PAI-1 accumulating and HVSMC cell-density. A similar correlation between the levels of t-PA/cell and PAI-1/cell and the number of cells/cm^2 was found for SMC isolated from saphenous vein of different donors, and for SMC isolated from umbilical vein or left internal mammary artery. This indicates that it is a general effect, which is not specific for a type of vessel nor for a particular cell-isolation or donor.
The negative correlation between t-PA and PAI-1 accumulation and cell-density could be caused by several mechanisms. Regulation could be on the level of gene-expression, protein synthesis, protein secretion, or via protein uptake and degradation by the cell. When regulation was on the level of gene-expression we would have expected a *decrease* in t-PA and PAI-1 mRNA levels in high cell-density cultures as compared to low cell-density cultures. We found however an *increase* in the t-PA and PAI-1 mRNA levels in high-density cultures as compared to low density cultures. The expression of mRNA can therefore not be an explanation for the negative correlation between cell-density and t-PA or PAI-1 levels.
Because binding and uptake can also influence t-PA and PAI-1 accumulation, we investigated t-PA binding to, and receptor expression on high and low cell-density HVSMC. Several investigators have shown that t-PA and t-PA:PAI-1 can bind to SMC (15,16) via two members of the low-density lipoprotein (LDL)-receptor family, the very low-density lipoprotein (VLDL)-receptor and the low density lipoprotein receptor related protein/α_2-macroglobulin receptor (LRP). These multi-ligand endocytosis receptors bind lipoproteins, toxins, proteinases and proteinase-inhibitor complexes and mediate their internalisation. Both LRP and VLDL-receptor are able to bind and internalise t-PA, pro-u-PA and plasminogen activator:PAI-1 complexes (11-13). By mediating the internalisation of u-PA, t-PA or their PAI-1 complexes, LRP and the VLDL-receptor can indirectly influence plasminogen activator activity. Recently, also another receptor for t-PA was described in human SMC (18). Whereas LRP and VLDL-receptor most efficiently bind t-PA:PAI-1 complexes, this as yet unidentified receptor only binds t-PA, with a K_d of about 25 nM.
We show that t-PA is able to bind to both high and low cell-density HVSMC, with a similar K_i value of 0.8 nM. However, low-density cultures show about 7 times more t-PA binding-sites than high-density cultures on a per cell basis. To examine whether t-PA bound to SMC via members of the LDL-receptor family, we competed for the binding of t-PA to high and low-density cultures with the LDL-receptor family antagonist RAP. RAP was able to competitively inhibit 60% of the binding of t-PA to HVSMC only in high cell-density

HVSMC. These results suggest that there are differences in the amount and type of receptors between high and low cell-density cultures. While t-PA binding-sites on low-density cells are mainly of a RAP-insensitive type (possibly cell-associated PAI-1), t-PA binds to high-density cells to both LDL-receptor family members as well as to RAP-insensitive receptors.
Association and degradation of t-PA in high-density cultures could be inhibited completely by RAP. This indicates that although part of the binding-sites in high-density cultures is of a RAP-insensitive type, all of the uptake and degradation of t-PA occurs via LRP and/or the VLDL-receptor. Association in low cell-density HVSMC could be inhibited for 40% by GST-RAP. This indicates that although the majority of binding-sites is of a RAP-insensitive type, association to low density cells is significantly mediated by LRP and/or VLDL-receptor.
When RAP was added to high and low-density HVSMC, only in high-density cultures an increase in the accumulation of endogenous t-PA was observed. The differences in the amount and type of receptors for t-PA between high and low-density cultures, and the differences in the effects of RAP indicate that these differences could be the cause of the cell-density dependent accumulation of t-PA and PAI-1.
We then examined if there were differences in the expression of LRP and/or the VLDL-receptor. We show that both high and low cell-density HVSMC express LRP and VLDL-receptor. Interestingly, the mRNA expression of VLDL-receptor is higher in high cell-density cultures, compared to low cell-density cultures. This suggests that this receptor plays a role in the cell-density dependent binding and association of t-PA. Several mechanisms might be responsible for the regulation of VLDL-receptor expression by cell-density. For example, at high cell-density, cells could secrete activators of VLDL-receptor expression, or at low cell-density, cells could secrete inhibitors. However, when we added 24 h conditioned medium of low density cells for 24 h to high density cells or when we added 24 h conditioned medium of high density cells for 24 h to low density cells, we found no effect on the VLDL-receptor mRNA level (results not shown). This suggests that another mechanism for the regulation of VLDL-receptor expression exist, possibly via cell-cell contacts.
The regulation of LDL-receptor family members by cell-density could be a more general mechanism. Recently, in the human breast cancer cell line BT-20 it was shown that there is a significant upregulation of LRP expression by increased cell density (19). In our experiments we could however not detect any differences in the amounts of LRP-protein between high and low cell-density SMC cultures. This suggests that in this case not LRP but other t-PA receptors, e.g. the VLDL-receptor, are most important. In cultured rat aortic SMC, a novel mechanism for the regulation of LRP activity has been described. Stimulation of SMC with

PDGF-BB and EGF did not increase LRP mRNA or protein levels (20), but instead redistributed LRP to the cell surface, resulting in an increased uptake and binding capacity. Via a similar mechanism, also cell-density may influence the redistribution of LRP to the cell surface.

In a previous study we showed that the addition of RAP could inhibit SMC migration and invasion (5). Members of the LDL-receptor family can play an important role in the clearance of plasminogen activator:PAI-1 complexes and excessive amounts of t-PA from the local environment, thereby regulating the proteolytic activity on the cell-surface. In conclusion, our experiments suggest that the cell-density dependent accumulation of t-PA and PAI-1 in SMC cultures is mediated by a different uptake via members of the LDL-receptor family, most probably via the VLDL-receptor. In this way cell-density could indirectly modulate migration and invasion of SMC.

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Chapter 6

Comparison of the plasminogen activation system in migration and invasion of human smooth muscle cells and endothelial cells *in vitro*

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Submitted

Summary

A role for the plasminogen activator (PA) system in vascular remodelling has been suggested from various studies using animal models or cell cultures. The PA-system is involved in neointima formation and smooth muscle cell migration after vascular trauma in animal models and formation of capillary-like tubular structures by bovine and human microvascular endothelial cells in a fibrin matrix. However, conflicting information exists regarding the involvement of plasminogen activators in lateral migration of endothelial cells.

In the present study we investigated the possible involvement of the plasminogen activation system in these processes in more detail using *in vitro* models of lateral and/or invasive migration of human smooth muscle cells and endothelial cells.

Lateral migration of human smooth muscle cells in wounded cultures could be inhibited by approximately 40 % when plasmin was inhibited, u-PA or t-PA activity was neutralised by specific antibodies or receptor binding of u-PA was inhibited by soluble receptor. A similar interference with plasminogen activation had no effect on lateral migration of human endothelial cells in wounded cultures or the lateral migration of human endothelial cells on fibrin. Invasion of smooth muscle cells through Matrigel could also be inhibited when plasmin was inhibited, specific antibodies neutralised u-PA or t-PA activity or receptor binding of u-PA was inhibited by soluble receptor. Invasive migration of human microvascular endothelial cells during formation of capillary-like tubular structures in a fibrin matrix was markedly inhibited in the presence of aprotinin (87%), antibodies against u-PA (67%) or soluble u-PA receptor (45%), but not in the presence of antibodies against t-PA.

We demonstrate that u-PA and t-PA mediated plasminogen activation is involved in the regulation of both lateral and invasive migration of human smooth muscle cells, whereas for human endothelial cells only the invasive migration appears to be plasmin mediated and lateral migration is plasmin independent. This indicates that inhibition of plasmin/plasminogen activator activity to prevent smooth muscle cell migration after balloon angioplasty will have no adverse effects on re-endothelialisation of the denuded area.

Introduction

Pericellular proteolysis plays a crucial role in the regulation of cell migration and invasion in general. In many pathophysiological processes related to the vascular system, such as angiogenesis or the intima thickening of atherosclerosis and restenosis, proteolytic enzymes are thought to mediate cell migration and tissue remodelling. The main enzyme systems involved are the plasminogen activator/plasmin system and the matrix metalloproteinase

system. The most important components of the plasminogen activation (PA) system are urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA), their type-1 (PAI-1) and type-2 (PAI-2) inhibitors and a cellular u-PA receptor (u-PAR). u-PA, especially when bound to its specific cell surface receptor (u-PAR), is thought to be involved in tissue remodelling and cell migration processes (1). This receptor not only localises the proteolytic activity (2, 3), but also enhances the conversion of pro-u-PA to the active two-chain u-PA (4, 5), possibly by close interaction with receptor-bound plasminogen (6).

Evidence for a role of the PA/plasmin system in vascular remodelling has been provided by several studies, both *in vitro* and *in vivo*. Inhibition of activity of plasmin or the plasminogen activators u-PA or t-PA resulted in a strong reduction of smooth muscle cell migration *in vitro* (7-9). *In vivo* experiments show that after balloon injury of the rat carotid artery a rapid increase of u-PA and t-PA levels occurred as an initial step of neointima formation (10, 11). Furthermore, inhibition of plasmin activity using tranexamic acid resulted in a reduction of the neointima formation after balloon injury *in vivo* (12). Analysis of neointima formation after vascular trauma in mice with a deficiency in PAI-1 shows a significant increase in the rate of neointima formation and neointimal cell accumulation (13). Mice deficient in u-PA show a significant decrease in neointima formation, whereas deficiency in t-PA had no effect on the neointima formation (14).

While ample evidence exists for the involvement of plasmin and plasminogen activators in the migration of smooth muscle cells, apparently conflicting information exits regarding the involvement of plasmin and plasminogen activators in migration and invasion of endothelial cells. Schleef and Birdwell (7) reported that inhibition of plasmin had no effect on lateral migration of bovine aortic endothelial cells in wounded cultures. In u-PA and t-PA deficient mice re-endothelialisation after vascular wounding was not affected (14). On the other hand, evidence for the involvement of u-PA, its receptor uPAR and its inhibitor PAI-1 in the regulation of the first steps of angiogenesis, i.e. local proteolytic remodelling of the extracellular matrix and migration of endothelial cells has been provided by *in vitro* experiments. u-PA activity is induced in bovine microvascular endothelial cells migrating from the edges of a wounded monolayer *in vitro* (15). These migrating cells displayed an increase in mRNA for u-PA, u-PAR and PAI-1 (16, 17). Furthermore, formation of tube-like structures in fibrin matrices by human microvascular endothelial cells is strongly reduced in the presence of an inhibitor of plasmin activity, antibodies against u-PA, and interference in the receptor binding of u-PA (18). This suggests a direct involvement of the u-PA/plasmin

system in invasive endothelial cell migration and capillary tube formation while its involvement in lateral migration, such as occurs during re-endothelialisation of the luminal surface of large vessels, is not clear.

In the present study we have tested this hypothesis and investigated the different role of the plasminogen activation system in the lateral migration and the invasive migration of both human smooth muscle cells and endothelial cells *in vitro*. We demonstrate that u-PA and t-PA mediated plasminogen activation is involved in the regulation of both lateral and invasive migration of human smooth muscle cells, whereas for human endothelial cells only the invasive migration appears to be plasmin mediated and lateral migration is plasmin independent.

Materials and Methods

Cell Culture

Human foreskin microvascular endothelial cells (HMVEC) and human umbilical vein endothelial cells (HUVEC) were isolated, cultured and characterised as previously described(19-22). Cells were cultured on fibronectin-coated dishes in M199 supplemented with 20 mmol/L HEPES (pH 7.3) (Flow Labs., Irvine, Scotland), 10% (v/v) human serum (local blood bank), 10% (v/v) heat-inactivated newborn calf serum (NBCS) (GIBCO BRL, Gaithersburg, MD), 150 μg/mL crude endothelial cell growth factor, 5 U/mL heparin (Leo Pharmaceutics Products, Weesp, The Netherlands), penicillin (100 IU/mL)/streptomycin (100 μg/mL) (Boehringer Mannheim, Mannheim, FRG) at 37°C under 5% CO_2/95% air (v/v) atmosphere.

Human umbilical vein smooth muscle cells (HUVSMC) were isolated from umbilical veins after isolation of HUVEC. The veins were incubated with culture medium (Dulbecco's modified Eagle medium (DMEM, GIBCO BRL, Paisley, Scotland) supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS), penicillin/streptomycin) containing 0.075% (w/v) collagenase (Worthington Biochemical Corp., Freehold, NJ, USA). After 45 minutes the incubation medium containing detached cells was flushed from the veins. Cells were washed and cultured on gelatine coated dishes in culture medium at 37°C under 5% CO_2/95% air (v/v) atmosphere. Cells were used for experiments between passage 4-6.

Migration assay

Confluent cultures of HUVSMC and HUVEC were mechanically wounded by removing cells by sticking the cells to dry Millipore filter strips (0.2 μm MF filters). After wounding, the cultures were washed twice with phosphate buffered saline (PBS) to remove residual detached cells. Subsequently, the cultures were incubated in culture medium to which either no additives (control), aprotinin (100 KIU/mL), a serine protease inhibitor which inhibits plasmin, or neutralising antibodies against t-PA(23), u-PA(24) or pre-immune IgGs were supplemented (50 μg/mL for endothelial cells and 125 μg/mL for smooth muscle cells). Cells were also incubated in medium containing 0.5 μg/mL human soluble u-PAR produced by transformed CHO cells (kindly provided by Dr. U. Weidle)(25).

Migration of the cells into the wounded area was followed by means of phase contrast microscopy over a period of 48 h and quantified by counting the cells that migrated into the denuded area. Migration of endothelial cells was also quantitatively analysed by determination of the distance the cells migrated into the denuded area in 24 h in multiple wounded cultures. Confluent cultures of HMVEC and HUVEC were grown for 24 h in the absence of crude endothelial cell growth factor. Then the cultures were wounded with a Plexiglass comb to create concentric circular denuded areas. After washing the cultures with medium to remove the detached cells, cultures were incubated for 24 h in medium containing 10% (v/v) human serum and 2.5 ng/mL bFGF (Boehringer Mannheim, Mannheim, FRG). Cultures were subsequently washed, fixed with 70% (v/v) ethanol for 10 min and stained with 0.2% (v/v) crystal violet. Migration was analysed using a Nikon FXA microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software.

Smooth muscle cell or endothelial cell invasion through Matrigel

Invasion of HUVSMC was assayed using a Transwell system with Matrigel coated polycarbonate filters (8 μm pore size, Costar, Cambridge, MA). Matrigel (Collaborative Biomedical Products, Bedford, MA) was layered onto the filter (14 μg/well) and allowed to dry at room temperature. Matrigel was hydrated with DMEM, penicillin/streptomycin before use. Cultured HUVSMC were trypsinised and suspended at a concentration of 4×10^5 cells/mL in DMEM, penicillin/streptomycin supplemented with 0.15 μmol/L plasminogen. A volume of 100 μL of cell suspension was placed in the upper chamber, and a volume of 600 μL of DMEM, penicillin/streptomycin supplemented with 0.15 μmol/L plasminogen and 10% (v/v) FBS as chemoattractant was placed in the lower chamber. To both lower and upper chambers either aprotinin (100 KIU/mL), rabbit anti-human u-PA IgG (125 μg/mL), rabbit anti-human t-PA IgG (125 μg/mL) or no additives (control) were added. After 24 h of incubation at $37^{O}C$ in 5% CO_2/95% air (v/v) the cells were fixed with 2.5% (v/v) glutaraldehyde. Matrigel and cells on the upper side of the filter were scraped off with a cotton swab, and cells on the lower side of the filter were stained with 2 g/L crystalviolet. Invasion was determined by counting the cells detected in microscopic fields (magnification x 200, n=24).

For analysis of the invasive migration through Matrigel of HUVEC, 4×10^5 cells were seeded in a similar way onto Matrigel coated filters in M199 supplemented 10% (v/v) FBS. M199 containing 10% (v/v) FBS and 10 ng/mL bFGF was added to the lower chamber as a chemoattractant. The invasive migration of HUVEC through Matrigel was analysed as described above.

In vitro angiogenesis model

Human fibrin matrices were prepared by addition of 0.1 U/mL thrombin to a mixture of 5 U/mL factor XIII (Behringwerke, Marburg, Germany), 2 mg fibrinogen, 2 mg Na-citrate, 0.8 mg NaCl and 3 μg plasminogen per mL M199 medium. 300 or 600 μL of this mixture was added to the wells of 48 or 24-well plates, respectively. After clotting at 37°C, the fibrin matrices were soaked with M199 supplemented with 10% (v/v) human serum and 10% (v/v) NBCS for 2 h at 37°C to inactivate the thrombin. Highly confluent endothelial cells were detached and seeded in a 1:1 split ratio on the fibrin matrices and cultured for 24 h in M199 medium supplemented with 10% (v/v) human serum, 10% (v/v) NBCS, and penicillin/streptomycin. Then the endothelial cells were cultured in medium containing 50 ng/mL bFGF, 100 ng/mL $VEGF_{165}$, 4 ng/mL TNFα (Biogent, Gent, Belgium) for 8-10

days, and the culture medium was collected and replaced every two or three days. Invading cells and the formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix were analysed by phase contrast microscopy and, after fixation of the matrices, by histological examination. The total length of tube-like structures was measured using a Nikon FXA microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software. Inhibition experiments were performed by the addition either of 100 KIU/mL aprotinin, 0.5 μg/mL soluble u-PA receptor, rabbit polyclonal anti-t-PA or anti-u-PA (50 μg/mL) to the culture medium.

Migration of endothelial cells over a fibrin matrix

Endothelial cells (HMVEC) were seeded in a 1:3 split ratio onto round glass coverslips that were coated with gelatine and cultured until they reached confluence. When HMVEC cultures reached confluence, they were put for 2-3 days on crude endothelial cell growth factor-free culture medium.
Fibrin matrices were produced by addition of 0.1 U/mL thrombin (Leo Pharmaceutics Products, Weesp, The Netherlands) to a mixture of 2 mg fibrinogen (Chromogenix AB, Mölndal, Sweden) per mL medium. 250 μL of this mixture was added to 12 well plates. After clotting at 37°C, the fibrin matrices were soaked with M199 supplemented with penicillin/streptomycin, 10% (v/v) human serum and 10% (v/v) NBCS, for 2 h at 37°C to inactivate the thrombin.
The glass coverslips with the confluent cells on top were carefully layered onto the fibrin. Endothelial cells were incubated in M199 containing 10 ng/mL bFGF, 2.5 ng/mL TNFα, 10% (v/v) NBCS, 10% human serum and penicillin/streptomycin. Cells were allowed to migrate onto the fibrin layer for 48 h, then the cells and fibrin layer were fixed with 2.5% (v/v) glutaraldehyde. Inhibition experiments were performed by the addition of either 100 KIU/mL aprotinin, 1 μg/mL soluble u-PA receptor, a rabbit polyclonal anti-t-PA (125 μg/mL), a rabbit polyclonal anti-u-PA (125 μg/mL), or a control pre-immune IgG (125 μg/mL) to the culture medium.

In vitro invasion of smooth muscle cells through fibrin

Invasion of smooth muscle cells through fibrin was assayed using Transwell chambers with fibrin coated polycarbonate filters. Fibrin matrices were produced by addition of 0.1 U/mL thrombin to a mixture of 2 mg fibrinogen/mL DMEM penicillin/streptomycin. 100 μL of this mixture was added to the Transwell chambers. After clotting at 37°C, the fibrin matrices were soaked with DMEM supplemented with 10% (v/v) FCS for 2 h at 37°C to inactivate the thrombin. Cultured smooth muscle cells were put on serum-free culture medium overnight. Cells were trypsinised and washed twice with DMEM, penicillin (100 U/mL)/streptomycin (100 μg/mL), 10% (v/v) FBS to inactivate the trypsin. Cells were suspended at a concentration of 4 x 10^5 cells/mL in serum-free culture medium supplemented with 0.15 μmol/L plasminogen. Subsequently a volume of 100 μL cell suspension was seeded in the upper chamber, and invasion was analysed as described above for Matrigel coated Transwell filters.

Histological Analysis

After overnight fixation at 4°C with 10 % (v/v) formaldehyde and 0.1% (w/v) amidoblack 10B, the fibrin matrices were washed three times in water, dehydrated by a graded series of ethanol and embedded in glycol methacrylate as described(18). The embedded matrices were cut (3 μm)

perpendicularly to the surface of the matrix sheet, stained with haematoxylin and analysed by light microscopy.

Statistical analysis

Data were expressed as mean ± S.E.M. Analysis of the statistical significance of the difference in the number of cells migrated into the denuded areas was performed with the one-way ANOVA analysis followed by a Fisher's Least Significant Difference test. Statistical significance of differences in length of the capillary tubes formed were tested by one-way ANOVA analysis followed by Dunnett's Multiple Comparisons Tests and accepted at $p<0.05$.

Results

Involvement of plasminogen activators in lateral migration of human smooth muscle cells

To study the role of plasminogen activators in lateral migration of human smooth muscle cells, confluent cultures of human umbilical vein smooth muscle cells (HUVSMC) were wounded by stripping the cells from their extracellular matrix using Millipore filterstrips. By this treatment the cells are removed, while their extracellular matrix remains attached to the culture dish. Migration into the denuded area after wounding the cultures was followed during 48 h (Figure 1) and quantified by counting the cells migrated into the denuded area. In the presence of the plasmin inhibitor aprotinin (100 KIU/mL) migration was inhibited to 61 ± 5 % after 48 h as compared to the control cultures (Figure 2). Specific inhibitory antibodies against u-PA reduced the number of migrated cells to 58 ± 3 % of control values and antibodies against t-PA to 69 ± 6 % of control values. Addition of soluble u-PAR to the culture medium, to prevent u-PA-binding to its cellular receptor, had a similar inhibitory effect on the migration as antibodies to u-PA (Figure 2).

Invasion of human smooth muscle cells in a basement membrane matrix

Next it was examined whether plasmin, u-PA, t-PA, and u-PAR play a role in invasive migration of smooth muscle cell invasion through an extracellular matrix barrier (Matrigel). Smooth muscle cell invasion was determined after 24 h in a Transwell system by quantifying the number of cells on the lower side of Matrigel-coated filters. Aprotinin (100 KIU/mL) reduced the number of cells invading through Matrigel to 20 ± 3% of control. Antibodies to u-PA inhibited smooth muscle cell invasion to 32 ± 5% of control, while antibodies to t-PA inhibited invasion to 53 ± 3% of control. Soluble u-PAR reduced the number of invaded cells to 52 ± 4% of control (Figure 3).

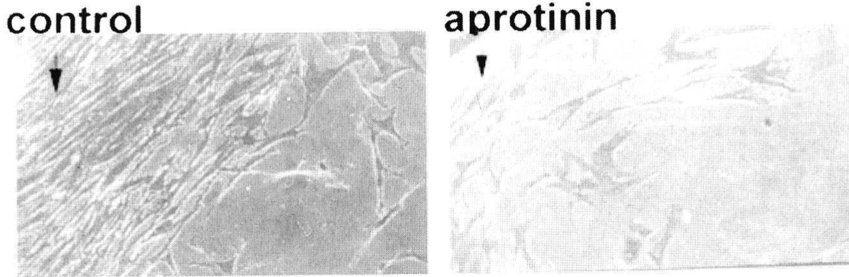

Figure 1. Migration of smooth muscle cells
Confluent cultures of human umbilical vein smooth muscle cells (HUVSMC) were wounded and subsequently re-incubated in the absence or presence of 100 KIU/mL aprotinin. Migration of cells into the denuded area was monitored for 48 h by phase contrast microscopy and photographs of consecutive regions along the edge of the wound were taken for quantitative analysis. The results after 48 h from a representative experiment are shown (the arrow indicates the edge of the denuded area).

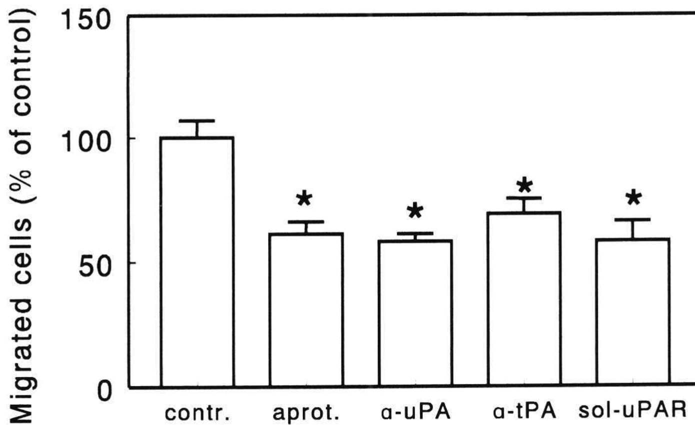

Figure 2.
Quantification of HUVSMC migration into the denuded area. Wounded cultures were incubated in the absence or presence of anti-t-PA IgG (125 μg/mL), anti-u-PA IgG (125 μg/mL), 2 μg/mL soluble u-PAR or 100 KIU/mL aprotinin. The number of cells migrated into the denuded area was determined after 48 h from consecutive photographs along the edge of the wound and expressed as percentage of control ± S.E.M. (n=9) (* indicates $p<0.01$). These results are also described in chapter 3, figure 2.

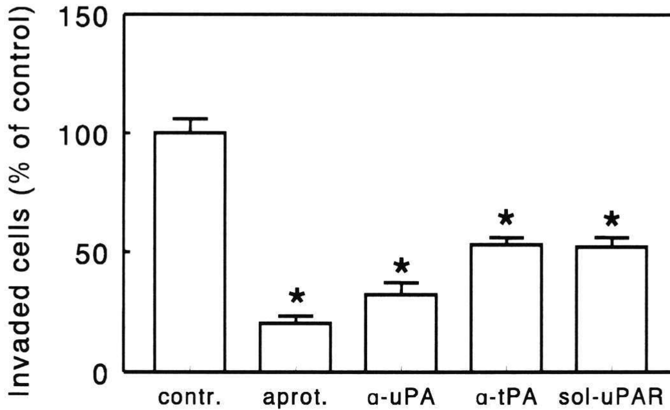

Figure 3
Transwell filters were coated with Matrigel. Smooth muscle cell suspension was placed in the upper chamber and DMEM containing chemoattractant (10% (v/v) FCS) was placed in the lower chamber. To both chambers 100 KIU/mL aprotinin, 125 µg/mL anti-u-PA IgG, 125 µg/mL anti-t-PA IgG, 3 µg/mL soluble u-PAR or no compound (control) was added. After 24 hours invasion was measured by counting the number of cells that migrated onto the underside of the filter. Data are expressed as percentage of control ± S.E.M. *$p<0.001$ ($n=24$).

Role of plasminogen activators in lateral migration of human endothelial cells in vitro

Similar lateral migration experiments as described above for human smooth muscle cells were performed with human endothelial cells, either HUVEC or HMVEC, to study the involvement of t-PA, u-PA, u-PAR and plasmin in endothelial cell migration in wounded monolayer cultures. Migration into the denuded area occurred, but in contrast to smooth muscle cells, no inhibitory effect on endothelial cell migration could be observed for any of the inhibitors used (Figure 4A). The inhibitory agents affected neither the number of cells that accumulated at various time points in the denuded area, nor the distance which the cells had migrated (Figure 4B).

Role of u-PA, u-PA receptor (u-PAR) and plasmin during in vitro angiogenesis

To investigate the involvement of u-PA, u-PAR and plasmin in invasive migration of human endothelial cell during angiogenesis first the invasive migration through Matrigel-coated Transwell filter was analysed. Unfortunately no invasive migration of HUVEC seeded on Matrigel-coated filters could be detected. As an alternative approach invasive migration was studied during the tube formation *in vitro* by HMVEC or HUVEC cultured on three-dimensional fibrin matrices in the presence of bFGF, $VEGF_{165}$ and TNFα (18). Formation of

tubular structures in the fibrin matrix started after 4 to 5 days of culture and the growth of these tubular structures was optimal at day 8 to 10 after culture of the endothelial cell monolayers. After 8 to 10 days of culture, invading cells and the formation of tubular structures of endothelial cells in the three-dimensional fibrin matrix were analysed by phase contrast microscopy and, after fixation of the matrices, by histological examination of cross-sections. Histological analysis of the cross-sections perpendicular to the surface of the matrix showed that these capillary-like structures were located in the fibrin matrix, underneath the endothelial cell monolayer. The capillary-like structures consisted of HMVEC surrounding a lumen (Figure 5).

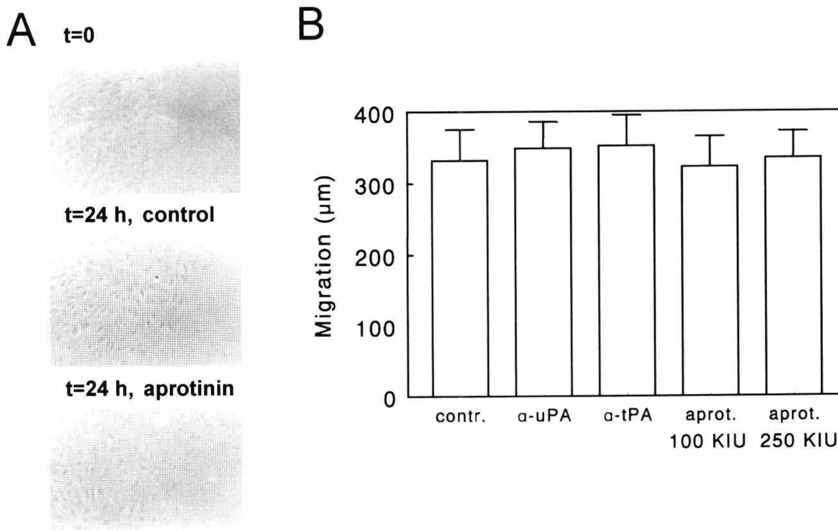

Figure 4
Confluent cultures of human umbilical vein endothelial cells (HUVEC) were wounded using either (**A**) a filter-strip and subsequently re-incubated in the absence or presence of 100 KIU/mL aprotinin. Migration of cells into the denuded area was monitored for 24 h by phase contrast microscopy and representative photographs are shown. Alternatively HUVEC cultures were wounded using a Plexiglass comb (**B**) and migration was quantified in cultures incubated with antibodies against u-PA, or t-PA, or with aprotinin (100 and 250 KIU/mL).

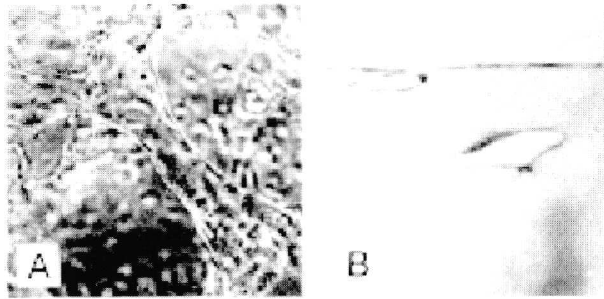

Figure 5
Human microvascular endothelial cells (HMVEC) cultured on the surface of a three-dimensional fibrin matrix in M199 medium supplemented with 10% human serum, 10% NBCS and bFGF (50 ng/mL), $VEGF_{165}$ (100 ng/mL), and TNFα (4 ng/mL) formed tube-like structures during a 10 days culture period. Tube-like structures were visualized by phase contrast photomicroscopy (**A**) the plane of focus is beneath the endothelial surface monolayer. Histological examination of 3 μm cross-sections perpendicular to the matrix surface was performed (**B**). Lumina are surrounded by endothelial cells, stained by haematoxylin.

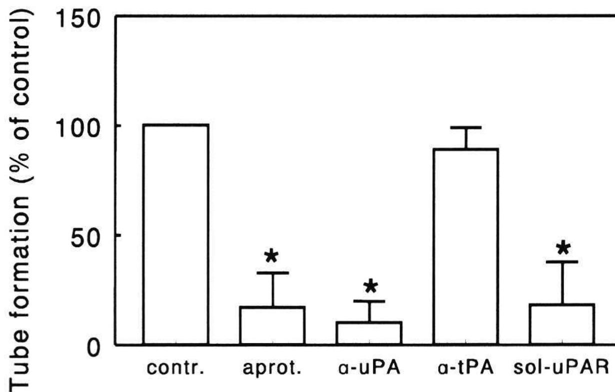

Figure 6
HMVEC were cultured on the surface of a three-dimensional fibrin matrix in M199 medium supplemented with 10% human serum, 10% NBCS and bFGF (50 ng/mL), $VEGF_{165}$ (100 ng/mL), and TNFα (4 ng/mL) with or without blocking antibodies against human u-PA, 0.5 μg/mL soluble u-PA receptor, or 100 KIU/mL aprotinin. After 10 days of culture, phase contrast photomicrographs were taken and the total length of tube-like structures was measured using a still video camera and an image analyser. The data represent mean length/microscope field ± S.E.M. of triplicate wells, and are representative for two experiments. * $p<0.05$.

Both antibodies against u-PA and soluble u-PAR inhibited the formation of tubular structures as determined by quantification of the total tube length (inhibition to 10 and 18% of control values, respectively, Figure 6). Addition of aprotinin also inhibited the total tube length to approximately 17% of control, indicating that plasmin activity, which is probably generated by u-PA, is also involved in the formation of tubular structures in the fibrin matrix. When fibrin matrices were made using plasminogen-depleted fibrinogen, no ingrowth of endothelial cells and formation of tubular structures was observed (data not shown).

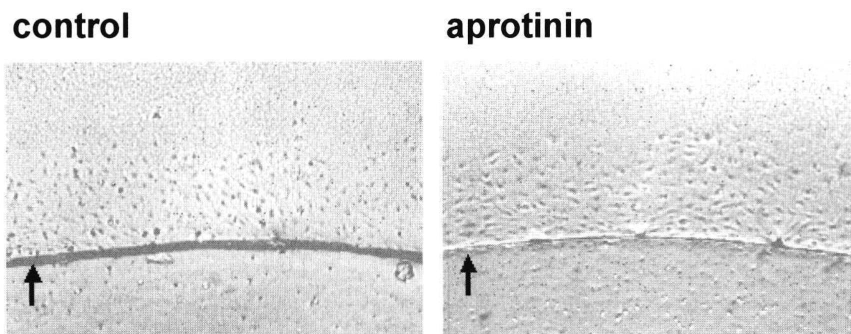

Figure 7
HMVEC were cultured on glass-coverslips until they reached confluence. Then the coverslips were put on a thin fibrin layer, and the endothelial cells were subsequently reincubated in the absence or presence of 100 KIU/mL aprotinin. Migration of cells into the denuded area was monitored for 48 hours by phase contrast microscopy and photographs of consecutive regions along the edge of the wound were taken for quantitative analysis. The results after 48 h are shown (the arrow indicates the edge of the glass-coverslip and the direction of endothelial cell migration).

Migration of human endothelial cells over a fibrin matrix.

Lateral movement of endothelial cells occurs in vivo over a damaged surface, which usually is covered with fibrin, such as happens when endothelial cells overgrow a mural thrombus. Therefore we also studied the migration of HMVEC cells from gelatine-coated glass-coverslips onto a fibrin matrix (Figure 7). Endothelial cells migrated rapidly onto the fibrin matrix. This process was also not inhibited by anti-u-PA, anti-t-PA, soluble-uPAR or aprotinin (Figure 8).

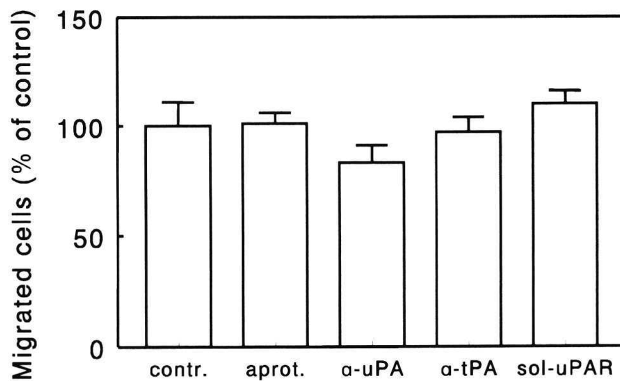

Figure 8
Quantification of HMVEC migration onto the fibrin layer. Endothelial cell-cultures on glass-coverslips were put onto a fibrin-layer and were incubated in the absence or presence of anti-t-PA IgG (125 μg/mL), anti-u-PA IgG (125 μg/mL), pre-immune IgG (125 μg/mL), 2 μg/mL soluble u-PAR or 100 KIU/mL aprotinin. The number of cells migrated into the denuded area was determined after 48 h from consecutive photographs along the edge of the wound and expressed as percentage of the control ± S.E.M.

Invasion of human smooth muscle cells into a fibrin matrix

We also studied the invasion of smooth muscle cells into a three-dimensional fibrin matrix that was similar to the fibrin matrix used for *in vitro* angiogenesis. Under control conditions, or in the presence of anti-u-PA, anti-t-PA or control antibodies, after an overnight incubation, the cells lost their ability to adhere to fibrin, and the fibrin matrix was almost degraded completely. Only with the addition of aprotinin in the assay, smooth muscle cells kept adhered onto the fibrin matrix and the fibrin was not degraded. Under these conditions it was not possible to detect invasive migration. At least these results indicate that smooth muscle cells produce such amounts of plasmin/plasminogen activator activity that the smooth muscle cells degrade rather than invade the fibrin-matrix.

Discussion

From the data presented here it can be concluded that both u-PA and t-PA mediated plasminogen activation is directly involved in lateral and invasive migration of human smooth muscle cells *in vitro*, as was demonstrated in the experiment with the wounded monolayer

cultures and Matrigel invasion (Figures 1-3). These data are in close concert with *in vivo* data. In animal models the putative role of plasminogen activation in smooth muscle cell migration is demonstrated in restenosis lesions induced by vascular injury. It has been shown by Clowes *et al.*(10), that after removal of the endothelium by balloon injury in the rat carotid artery, the expression of u-PA and t-PA is induced rapidly after injury. Furthermore, it has been demonstrated that plasmin generation was necessary for migration of smooth muscle cells, since addition of tranexamic acid, an inhibitor of plasminogen activation, resulted in reduction of smooth muscle cell migration (12). Experiments with 'knock-out' mice, in which mice deficient in plasminogen or u-PA show a significant decrease in neointima formation after vascular trauma whereas mice deficient in PAI-1 show a significant increase, suggest a role for u-PA mediated plasminogen activation in smooth muscle cell migration in neointima formation (13, 14, 26). In t-PA deficient mice there was however no effect on neointima formation, suggesting that at least in mice t-PA is not involved in neointima formation (14).
After injury, u-PAR has been found polarised to the front of migrating smooth muscle cells (27). Here we show that both lateral migration and invasion through Matrigel of smooth muscle cells is markedly reduced in the presence of soluble u-PAR, a compound preventing the binding of u-PA to its cell-surface receptor. These data suggest that for human smooth muscle cells migration a similar role for cell surface-bound u-PA-mediated plasmin generation can be envisaged as for tumour cell migration (5, 28, 29). Experiments in u-PAR deficient mice, however, showed no effect on neointima formation as compared to normal mice (30). This might suggests that at least in mice, binding of u-PA to u-PAR is not required to provide sufficient pericellular u-PA mediated plasmin proteolysis to allow SMC migration into the vascular wound.
A most interesting observation is that for lateral migration of endothelial cells plasmin activity is not essential, although invasive migration through fibrin seems to be PA/plasmin dependent. Even lateral migration over a fibrin matrix is PA/plasmin independent since our observations were done with wounded monolayers in which the cells migrated over a residual matrix (which remained in the wounded area after detachment of the cells with Millipore filter strips), over the plastic culture dish (after scraping the cells with a comb), and over a fibrin matrix. A similar observation has been reported for bovine aorta endothelial cells (7). In this case plasmin inhibition by aprotinin had no effect on migration, although that for bovine microvascular endothelial cells an induction of expression of u-PA, PAI-1 and u-PAR mRNA has been observed upon wounding of these cultures (15-17). Also, in transgenic mice deficient in components of the plasminogen activation system, no differences in re-endothelialisation

were found after vascular injury (30, 31). These data suggest that there is a cell-type specific requirement of plasmin proteolysis for cellular migration.

Fibrin is a temporary repair matrix. In tissue repair, endothelial cells will usually grow into a fibrin matrix, often preceded by inflammatory cells. Endothelial cells cultured on three-dimensional fibrin matrices can be induced to form capillary-like tubular structures in the fibrin matrix (18, 32, 33). Here we demonstrate that u-PA, but not t-PA, mediated plasminogen activation is involved in this process. Tube-length was significantly reduced in the presence of aprotinin or u-PA antibodies, but not in the presence of t-PA antibodies. Plasminogen activation was mediated by receptor bound u-PA, because addition of soluble u-PAR also resulted in a reduction of tube-length.

Unfortunately, a direct comparison of smooth muscle cell invasion into a fibrin matrix with endothelial cell invasion was not possible due to technical problems. The high plasminogen activator activity of the smooth muscle cells resulted in the complete degradation of the fibrin matrix.

The observation that lateral migration of endothelial cells is not plasmin dependent, whereas both invasive and lateral migration of smooth muscle cells is, suggests that inhibition of plasmin activity possibly can be used as a therapeutic approach to inhibit neointima formation in denuded blood vessels without affecting the essential re-endothelialisation.

Acknowledgements

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Chapter 7

Immunohistochemical analysis of the expression of the plasminogen activation system and the matrix metalloproteinases in normal and atherosclerotic human vessels

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Submitted

Summary

In this study we examined the localisation of the plasminogen activation system and the matrixmetalloproteinases in normal human arteries, arteries with diffuse intimal thickenings and atherosclerotic arteries. We found that MMP-1, MMP-2, MMP-3, TIMP-1 and TIMP-2 did not stain differently in normal arteries, arteries with intimal thickening and atherosclerotic arteries. MMP-9 staining was, however, increased in atherosclerotic arteries as compared to normal arteries and arteries with diffuse intimal thickening. This suggests that the ultimate MMP-activity is increased in the intima of atherosclerotic arteries. For u-PA there were no differences between normal arteries, arteries with intimal thickening and atherosclerotic arteries. t-PA staining was decreased in the media of atherosclerotic arteries as compared to both normal and intima-thickened arteries. In contrast PAI-1 staining was elevated in the intima of atherosclerotic arteries as compared to the media. While u-PAR was elevated in the media of arteries with intima thickening, staining was weaker in the intima of atherosclerotic arteries, as compared to the intima of normal arteries. These results suggest that the ultimate plasminogen activator activity may be decreased in atherosclerotic arteries. We propose that because the MMP-activity may be upregulated, while the plasminogen activator activity may be downregulated in atherosclerotic arteries as compared to normal arteries, the MMP-system and the PA-system may play different roles in the process of atherosclerosis.

Introduction

Vascular smooth muscle cells play a fundamental role in neointima formation after vascular injury and in the development of arteriosclerosis. In the early stage of these processes, smooth muscle cells proliferate in the media. Subsequently, smooth muscle cells migrate from the media, through the internal elastic lamina, to the intima. Intimal thickenings are further formed by proliferation of smooth muscle cells in the intima and the deposition of excessive amounts of extracellular matrix components (1-3).

Proteolytic enzymes can regulate smooth muscle cell migration through the controlled proteolytic degradation of the extracellular matrix that encages these cells. Smooth muscle cells are known to produce several proteolytic enzymes, including members of the plasminogen activation (PA)-system and the matrix metalloproteinase (MMP)-system. Several studies have shown that these two enzyme systems are involved in the migration of smooth muscle cells (4-11).

The PA-system contains the pro-enzyme plasminogen, which is converted to the active enzyme plasmin by urokinase-type plasminogen activator (u-PA) or tissue-type plasminogen activator (t-PA). u-PA can bind to a specific receptor (u-PAR) on the cell surface, resulting in

pericellular proteolysis. Both plasminogen activators can be inhibited by their main physiological inhibitor PAI-1.

Plasmin can degrade several components of the extracellular matrix, including laminin, fibronectin, vitronectin, and proteoglycans. It can however also indirectly enhance extracellular proteolysis through the activation of latent pro-MMPs, such as proMMP-1 (interstitial collagenase), proMMP-3 (stromelysin), and proMMP-9 (gelatinase B). Activated MMPs, like MMP-3, can also enhance proteolysis by activating other proMMPs (12). The MMPs can be inhibited by tissue inhibitors of metalloproteinases (TIMPs), of which four are known.

In addition to the regulation of smooth muscle cell migration, proteolytic enzymes also play a role in macrophage infiltration and in controlling the synthesis and degradation of the connective tissue matrix. Increased protease activity could weaken atherosclerotic plaques, predisposing them to rupture and thrombosis.

Several investigators have studied the localisation of members of the PA-system or the MMP-system in normal and atherosclerotic arteries (13-26). Contradictory results concerning the staining intensity and localisation of members of these enzyme systems have however been found. There are also no studies that have examined both systems in the same vessels. In this study we therefore compared the localisation of the PA-system and the MMP-system in normal human arteries, arteries that have intima thickening and atherosclerotic arteries.

Materials and methods

Tissue specimens

Artery specimens from 13 persons were all obtained at autopsy, from the department of Pathology, St. Radboud, Nijmegen, the Netherlands. Specimens were rinsed in phosphate-buffered saline (PBS), submerged in Tissue-Tek (Miles Laboratories, Naperville, USA) and quickly frozen at -80°C. Sections of 6 μm thick were cut at -24°C and stored at -20°C.

Segments of 7 human abdominal aortas, 8 internal mammary arteries, and 5 coronary arteries were examined. The morphology of the vessels was studied with hematoxylin-eosin (HE) staining to determine if the vessels were normal, showed a diffuse intimal thickening, or were atherosclerotic. A vessel was defined as being normal when there was no intima, or a small intima. A vessel was defined as having a diffuse intimal thickening when a clear thickened intimal layer was visible. A vessel was defined as being atherosclerotic when there were (thick) intimal layers with necrotic regions and/or calcifications. In total, 4 aortas and 5 internal mammary arteries were defined as being normal. Furthermore, 2 internal mammary arteries and 2 coronary arteries were defined as having a diffuse intimal thickening, while 3 aortas, 1 internal mammary artery and 3 coronary arteries were defined as being atherosclerotic (table 1).

Case	Age (years)	Gender	Types of vessels studied
1	n.k.	n.k.	aorta, normal
			i.m.a., normal
			i.m.a., diffuse intimal thickening
2	n.k.	n.k.	aorta, atherosclerotic
3	71	F	aorta, normal
			i.m.a., normal
4	57	M	aorta, normal
			a.cor. diffuse intimal thickening
5	84	F	aorta, normal
6	82	M	aorta, atherosclerotic
			i.m.a., diffuse intimal thickening
7	71	M	aorta, atherosclerotic
			i.m.a., atherosclerotic
			a.cor., atherosclerotic
8	n.k.	n.k.	i.m.a., normal
9	n.k.	n.k.	i.m.a., diffuse intimal thickening
10	59	M	i.m.a., normal
11	60	M	i.m.a., normal
12	n.k.	n.k.	a.cor., atherosclerotic
13	50	F	a.cor., atherosclerotic

Table 1. The tissue specimen used in this study.
Human abdominal aorta (aorta), internal mammary artery (i.m.a.), coronary artery (a.cor) (F=female, M=male, n.k.= not known).

Antibodies

Polyclonal goat-anti-human melanoma t-PA antibody (#387), monoclonal anti-u-PA (B-chain) antibody (#3689), and monoclonal antibody directed against PAI-1 from HT-1080 cells (#380) were all from American Diagnostica Inc. (Greenwich, CT, USA). H2 monoclonal antibody, directed against human u-PAR was a kind gift from Dr. U. Weidle (Boehringer Mannheim, Penzberg, Germany). Monoclonal antibodies recognising MMP-1 (Ab-1), MMP-2 (Ab-1), MMP-3 (Ab-1), MMP-9 (Ab-1), TIMP-1 (Ab-1), and TIMP-2 (Ab-1) were all from Oncogene Research Products (Cambridge, MA, USA). All antibodies were used at a final concentration of 5 μg/mL.
For the identification of endothelial cells, CD31 (1:40) (CLB, Amsterdam, the Netherlands), a monoclonal antibody which recognises PECAM-1 was used. Smooth muscle cells were identified with a monoclonal anti-α-actin antibody (1:100) (asm-1, Boehringer Mannheim, Germany).

Immunohistochemistry

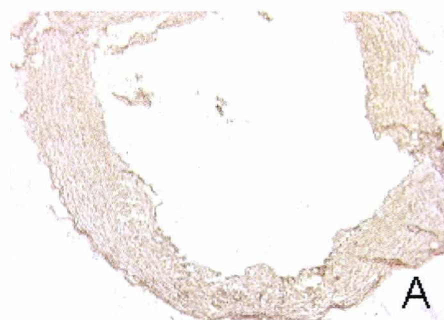
Immediately before use the sections were fixed in cold acetone (-20°C) for 10 minutes, and then air-dried for 1 h at room temperature. Sections were rehydrated, rinsed in PBS, and preincubated with 0.3% (v/v) H_2O_2 and 0.1% (w/v) sodium azide in PBS for 15 minutes to eliminate endogenous peroxidase activity.
Sections were incubated overnight at 4°C with the primary antibodies. Bound α-actin and PECAM-1 antibodies were detected using horseradish peroxidase (HRP) conjugated rabbit-anti-mouse antibodies

(RAMPO) (1:250) (DAKO A/S, Glostrup, Denmark). All other antibodies were detected via the indirect unconjugated peroxidase-anti-peroxidase technique. After the binding of RAMPO (to bound monoclonal antibodies) or HRP-conjugated goat-anti-rabbit antibodies (GARPO) (1:250) (DAKO A/S, Glostrup, Denmark) (to bound polyclonal antibodies) overnight at 4°C, sections were first incubated for 1 h at 37°C with goat-anti-rabbit serum (1:50) (Nordic, Tilburg, the Netherlands) and then for 1 h at 37°C with R/PAP (rabbit-peroxidase-anti-peroxidase complex) (1:500) (Nordic, Tilburg, the Netherlands) as an amplification step. All antibodies were diluted in PBS with 1% (w/v) bovine serum albumin, 1% (v/v) human serum (local blood bank), and 0.05% (v/v) Tween-20.
The sections were exposed for 8 minutes to 0.04% (w/v) diaminobenzidine tetrahydrochloride (DAB) in 0.05 mol/L Tris-maleate buffer (pH 7.6) with 0.006% (v/v) H_2O_2. The reaction was stopped in PBS. Sections were counterstained with hematoxylin. All sections were dehydrated in graded ethanol and mounted in mounting medium.
Control sections were incubated in PBS with 1% (w/v) bovine serum albumin, 1% (v/v) human serum and 0.05% (v/v) Tween-20, instead of the primary antibody. These sections showed no staining.

Quantification of staining
Immunocytochemical data were described qualitatively and semiquantitatively; no staining was recorded as 0; weak diffuse staining as 1; moderate staining as 2; good staining as 3; very strong staining as 4. For each antibody at least two serial sections per sample were investigated. Data are presented as median with interquartile range. Statistical analysis was performed with non-parametric one-way analysis of variance (Kruskal and Wallis). Statistical significance was accepted for $p<0.05$.

Results

Figure 1. Matrix metalloproteinase expression in a normal mammary artery and an atherosclerotic aorta →
An normal mammary artery (**A,C,E,G**) and part of an atherosclerotic aorta which has a clear intimal thickening with a necrotic core (**B,D,F,H**) are shown,. (**A,B**) α-actin (SMC). Strong staining in the normal artery and absence of staining in the necrotic core of the plaque and weak staining in the cap of the lesion in the atherosclerotic artery. (**C,D**) TIMP-1. Strong staining in the normal artery. In the atherosclerotic artery there is smooth muscle cell-associated staining in the media, and the cap of the intima. The fibrous a-cellular part of the lesion is staining diffuse. (**E,F**) MMP-2. Moderate staining in the necrotic core and the media in the atherosclerotic lesion. (**G,H**) MMP-9. Staining of the media and the necrotic core of the plaque in the atherosclerotic artery. All photomicrographs original magnification x20. a=adventitia, i=intima, m=media, n=necrosis.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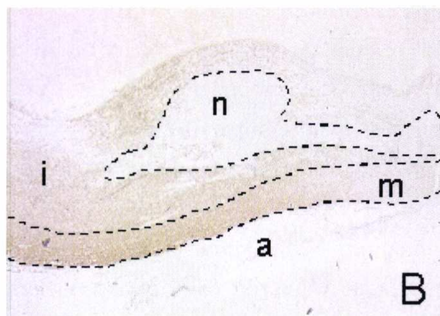
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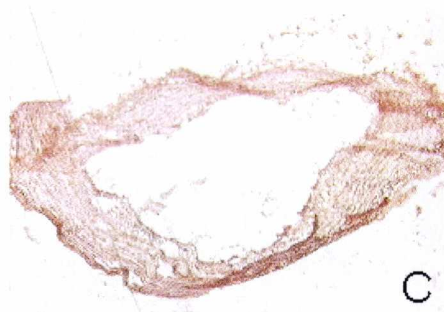
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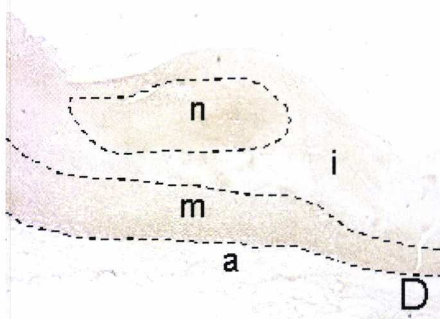
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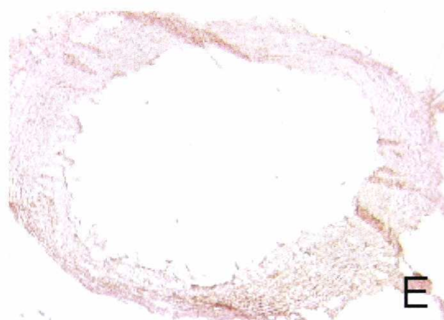
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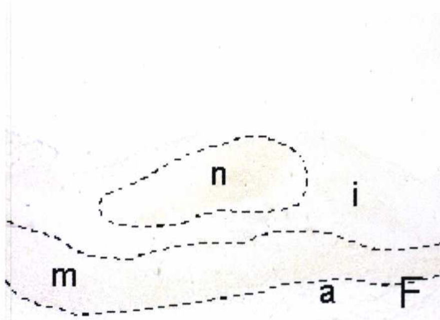
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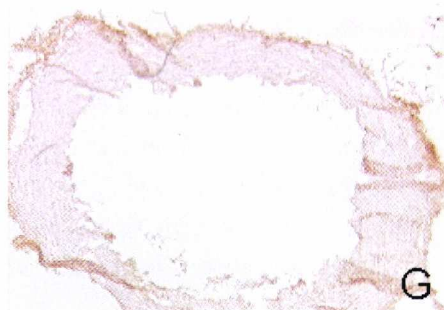
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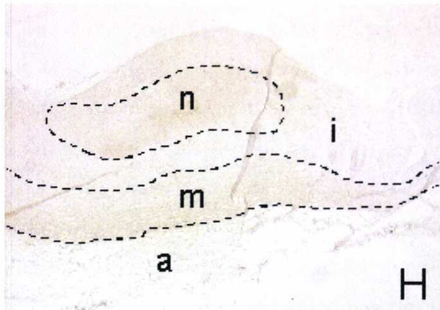
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Matrix metalloproteinases (MMPs)

MMP-2 staining was moderate to good in normal arteries and was smooth muscle cell-associated. Lumen endothelium and vasa vasorum also stained for MMP-2. Staining intensities were comparable in arteries with intimal thickenings or atherosclerotic lesions (table 2, figure 1F). In atherosclerotic lesions, however, staining was stronger around calcified areas.

Staining for MMP-3 was weak to moderate in normal arteries. Staining intensities were comparable in arteries with intimal thickening and with atherosclerotic lesions (table 2). In atherosclerotic arteries, however, there was also a clear strong MMP-3 staining near calcifications and in lumen endothelial cells (figure 3G).

In normal arteries and in arteries with an intimal thickening, MMP-9 staining was smooth muscle cell-associated and generally weak, while in atherosclerotic lesions there was a significantly stronger immunodetectable MMP-9 staining (figure 1H, table 2) in the media (as compared to both normal and intima-thickened arteries) and intima (as compared to arteries with intima thickening). Staining was also detected in endothelial cells in the lumen (figure 3H), in the vasa vasorum, and in atherosclerotic lesions in neomicrovessels (figure 3C,D).

Tissue inhibitors of metalloproteinases (TIMPs)

TIMP-1 staining was strong in normal arteries, arteries with diffuse intimal thickenings and atherosclerotic arteries and was smooth muscle cell-associated (table 2, figure 1D).

In normal arteries TIMP-2 staining was smooth muscle cell-associated and weak. Lumen endothelium showed moderate staining (figure 3F). Staining was similar in arteries with intimal thickening and with atherosclerotic lesions (table 2).

Plasminogen activators

In normal arteries there was a moderate staining for u-PA. Staining was similar in arteries with a diffuse intimal thickening and arteries with atherosclerotic lesions (table 2).

In normal arteries there was a good t-PA staining in the vasa vasorum and in smooth muscle cells. In arteries with a diffuse intimal thickening, t-PA staining was similar to that in normal arteries. In atherosclerotic arteries, staining was significantly weaker in the media as compared to the media of normal arteries or arteries with intimal thickening (table 2, figure 2D). Although there was no significant difference in staining intensity between the media and the intima in atherosclerotic arteries (table 2), t-PA staining was always very strong around calcificated areas and in neomicrovessels (figure 3A,B).

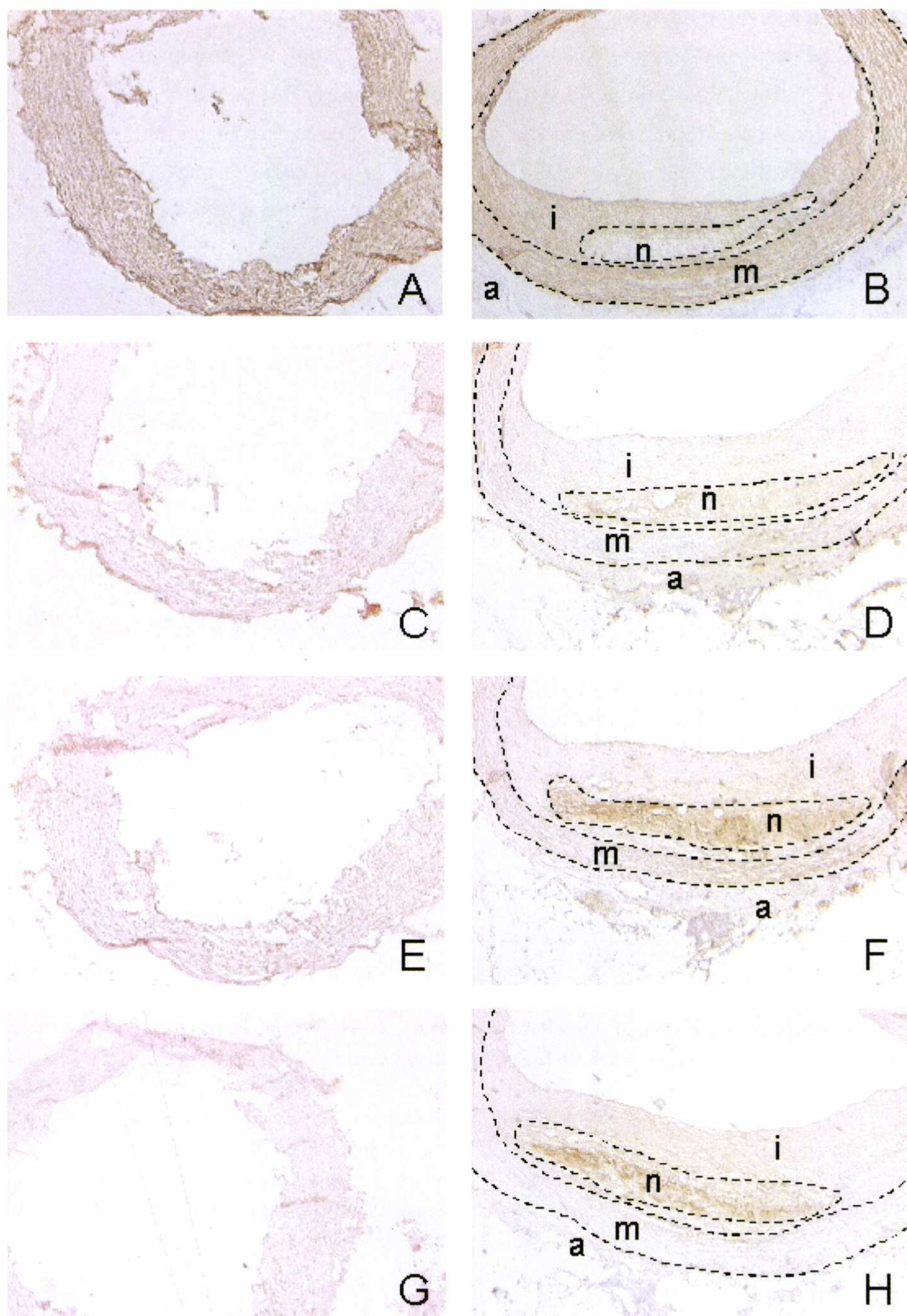
Plasminogen activator inhibitor-1 (PAI-1)

In normal arteries PAI-1 staining was weak (figure 2G). Staining was similar in arterial tissues with diffuse intimal thickenings. In atherosclerotic arteries, PAI-1 staining was somewhat weaker in the media of atherosclerotic arteries as compared to the normal media. PAI-1 staining was significantly stronger in the intima than in the media of atherosclerotic arteries (table 2), in particular around calcifications, in the necrotic core and at the base of the plaque (figure 2H).

		Normal	Intimal thickening	Atherosclerosis
MMP-1	Media	4 (3-4)	3 (1.5-4)	2 (1.5-3)
	Intima	1 (1-2)	2.5 (1-4)	3 (1.75-4)
MMP-2	Media	3 (3-3)	2.5 (1.5-3)	3 (1.75-3)
	Intima	3 (2.5-3)	1 (1-2)	1.5 (0.75-2.5)
MMP-3	Media	1 (1-2)	1.5 (1-2.5)	3 (0.5-3)
	Intima	1 (0.5-1)	1 (0.5-2)	1.5 (0.75-2.5)
MMP-9	Media	1 (0-1)	0.5 (0-1)	3 (2.5-3)*/**
	Intima	1 (1-1.5)	1 (0.5-1)	2.5 (1.75-3)**
TIMP-1	Media	4 (3-4)	4 (4-4)	4 (3.5-4)
	Intima	4 (3.5-4))	3.5 (3-4)	3.5 (2.75-3.5)
TIMP-2	Media	1 (1-2)	1 (1-1.5)	1 (1-1)
	Intima	1 (0.5-1.5)	1 (1-1.5)	2 (0 .5-2.5)
u-PA	Media	2 (1-2)	2 (2-2)	1 (1-1.75)
	Intima	1 (1-1.5)	2 (1.5-2)	2 (1-2.25)
t-PA	Media	3 (2-3)	3 (2.5-3.5)	1.5 (1-2)*/**
	Intima	3 (2.5-3)	2 (2-2.5)	1.75 (1-2.5)
PAI-1	Media	1 (1-2)	1.5 (0.5-2.5)	0.5 (0-1)*
	Intima	2 (1.5-2.5)	1.5 (0.5-3)	2.5 (2.25-3.25)#
u-PAR	Media	3 (3-4)	4 (4-4)*	3.5 (3-4)
	Intima	2 (2-3)	4 (4-4)	2 (1-2.5)**/#

Table 2. Semiquantitative analysis of members of the plasminogen activation system and the matrix metalloproteinase system detected by immunocytochemistry in the media and intima of normal arteries (n=9), arteries with diffuse intimal thickening (n=4) and atherosclerotic arteries (n=7)

Staining was evaluated in media and intima of abdominal aorta, coronary artery, and internal mammary artery. No staining was recorded as 0; weak diffuse staining as 1; moderate staining as 2; good staining as 3; very strong staining as 4. Results are presented as median (interquartile range). *$p<0.05$ as compared to normal. **$p<0.05$ as compared to intimal thickening. # as compared to media.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← **Figure 2. Plasminogen activation system in an atherosclerotic and a normal mammary artery.**
Part of an normal mammary (**A,C,E,F**) and of an atherosclerotic mammary artery with a clear intimal thickening and a necrotic core (**B,D,F,H**) is shown,. (**A,B**) α-actin staining for SMC. Strong staining in the normal mammary artery. There is absence of staining in the necrotic core of the plaque in the atherosclerotic artery. (**C,D**) t-PA. Staining in the necrotic core, in lumen endothelium, and strong staining in vasa vasorum in the atherosclerotic artery. (**E,F**) u-PAR. In normal artery uPAR is detectable in smooth muscle cells, vasa vasorum and in lumen endothelium. In the atherosclerotic artery there is immunolocalisation in the media, the necrotic core, the vasa vasorum and in lumen endothelium. (**G,H**) PAI-1. Strong staining in the necrotic core and good staining in the fibrous cap of the plaque in the atherosclerotic artery. All the photomicrographs original magnification x 20. a=adventitia, i=intima, m=media, n=necrosis.

u-PAR

In normal arteries, u-PAR staining was good to strong (figure 2E). u-PAR was immunocytochemically detectable in smooth muscle cells, vasa vasorum and in lumen endothelium. u-PAR staining was significantly stronger in the media of arteries with intimal thickenings than in the intima of normal arterial tissue and the intima of atherosclerotic arteries (table 2). In atherosclerotic arteries staining was weaker in the intima than in the media (table 2). In atherosclerotic lesions with a clear necrotic core, intima staining was stronger at the base of the plaque than in the surrounding tissue (figure 2F).

Discussion

Factors that regulate the synthesis and degradation of the extracellular tissue matrix are important in atherosclerosis and intima formation. A high proteinase activity can result in plaque instability and rupture of the plaque can occur. Proteinases can also play a role in the migration of cells, through localised proteolytic degradation of the extracellular matrix. Several studies have therefore investigated the expression of members of the matrix metalloproteinases or the plasminogen activation system in normal human vessels, and compared this with atherosclerotic lesions.

In normal arteries, these studies have shown a weak immuno-staining for MMP-1, MMP-3 and MMP-9 (13-16), and a positive immuno-staining for TIMP-2 (13,15). Literature has, however, produced contradictory results for MMP-2 and TIMP-1, ranging from no/very weak staining to positive staining (13-15). Several investigators have shown that, compared to normal arteries, atherosclerotic arteries have an increased expression for MMP-1, MMP-2, MMP-3, MMP-9 and TIMP-1 (13-15,17). However, for MMP-2 a report by Galis *et al.* (15) showed that there is a similar staining intensity in normal and atherosclerotic arteries. For TIMP-2 no differences between normal and atherosclerotic arteries have been found (13,15).

In this present study, we find that all tested MMP-s, TIMP-1 and TIMP-2 show positive staining in normal arteries. The staining intensities of MMP-1, MMP-2, TIMP-1, TIMP-2, and MMP-3 are not changed in arteries with diffuse intimal thickening or atherosclerotic arteries, but MMP-9 is increased in atherosclerotic plaques. These results imply that the ultimate MMP-activity is increased in the media and also in the intima of atherosclerotic arteries, and suggest that the MMP-system is involved in the atherosclerotic process

Several studies have examined the expression of the plasminogen activation system in normal and atherosclerotic vessels, but with contradictory results. Falkenberg *et al.* found no u-PA in normal arteries (18), while others (19-23) found positive staining. In normal arteries, Falkenberg detected t-PA only in the endothelial cells of the vasa vasorum (18). On the other hand, others have found t-PA in endothelial cells, associated with the vasa vasorum and in smooth muscle cells in normal arteries (19,22). As for t-PA there are also contradictory results about the localisation of PAI-1. One study found no or only very weak staining in normal arteries (24). In other studies PAI-1 was detected in the media and in both lumen and vasa vasorum endothelial cells (18,21,22,25). In atherosclerotic arteries, an increased expression of u-PA, t-PA, PAI-1 and u-PAR has been found (18-20,22,23,26).

In this study we find that u-PA, t-PA, PAI-1 and u-PAR all stain positively in normal arteries. For u-PA there were no differences between normal arteries, arteries with intimal thickening and atherosclerotic arteries. t-PA staining was decreased in the media of atherosclerotic arteries as compared to both normal and intima-thickened arteries. In contrast PAI-1 staining was elevated in the intima of atherosclerotic arteries as compared to the media. We also found, that while u-PAR was elevated in the media of arteries with intima thickening, staining was weaker in the intima of atherosclerotic arteries. These results together suggest that the ultimate plasminogen activator activity is downregulated in atherosclerotic arteries.

In part our observations are in contrast to the studies mentioned above. An explanation for this could be that atherosclerotic lesions are very heterogeneous in nature and the expression of proteolytic enzymes and inhibitors probably depends on the stage of the atherosclerotic process and the characteristics of the atherosclerotic lesion.

Infiltrating macrophages are usually found around the necrotic core. Several reports have shown that proteolytic enzymes are particularly strong expressed by macrophages located in the plaque. MMP-3, u-PA, t-PA and PAI-1 have been found colocalising with macrophages in the plaque, often in shoulder regions of necrotic cores or localised within the plaque core (13,15,19-23). In our study we also show a strong staining for MMP-3, u-PA, t-PA and PAI-1 and in addition for MMP-2, MMP-9, TIMP-1, and uPAR in and around the necrotic core in atherosclerotic plaques. Although we did not determine if our tissue specimens contain

regions of macrophage infiltration, it is likely that this strong staining is due to the presence of macrophages in these advanced lesions.

While the MMP level may be upregulated in atherosclerotic arteries, the plasminogen activator level may be downregulated. Immunological staining does not provide information about the actual enzyme activity, because both enzyme-systems may exist as pro-enzyme or in inactive forms.

Our results suggest that the MMP-system and the PA-system play different roles in the process of atherosclerosis.

Acknowledgements

We would like to acknowledge the financial support of the Netherlands Heart Foundation grant 93.154. The experiments described in this article would not have been possible without the help of Cilia Ferrier and colleagues from the Department of Pathology, St. Radboud, Nijmegen, the Netherlands, who collected the tissue specimens. Jacqueline Bastiaanse is gratefully acknowledged for performing the initial experiments and Eric Offermans for technical assistance.

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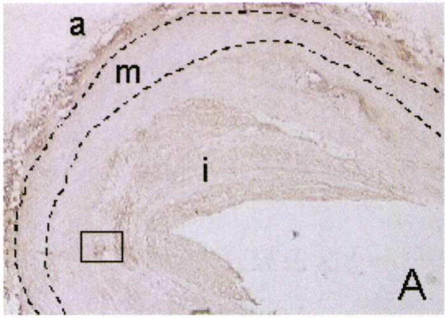
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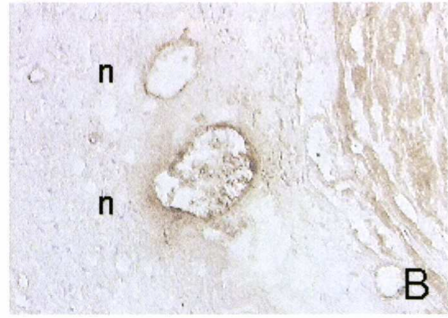
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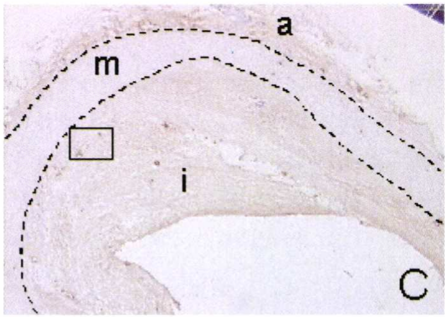
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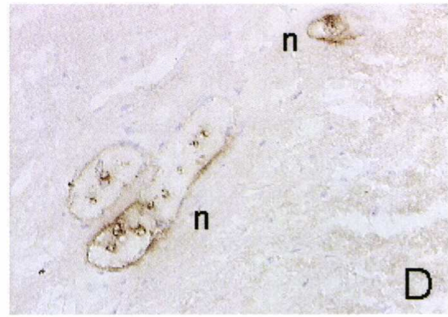
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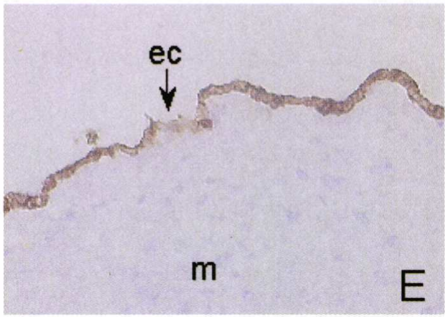
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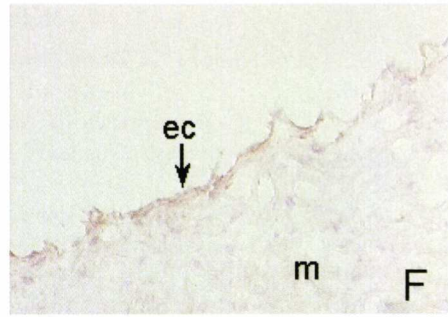
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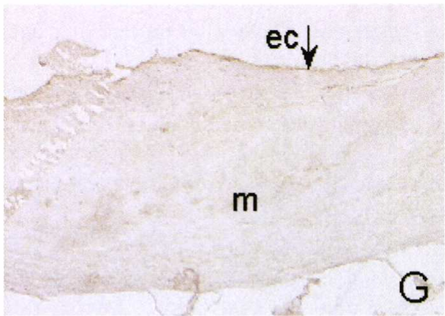
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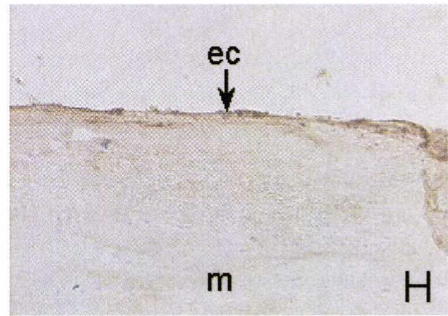
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← Figure 3. Matrix metalloproteinases and plasminogen activators in neomicrovessels and in lumen endothelial cells
(A) Immunolocalisation of t-PA in an atherosclerotic coronary artery (original magnification x 20) **(B)** Detail of (A). t-PA in neomicrovessels (original magnification x 100) of a coronary artery. **(C)** Immunolocalisation of MMP-9 in an atherosclerotic coronary artery (original magnification x 20). **(D)** Detail of (C). MMP-9 in neovessels (original magnification x 100). **(E)** PECAM-1 staining of lumen EC in a mammary artery (original magnification x 200). **(F)** Staining of TIMP-2 in lumen endothelium in the same vessel as (E) (original magnification x 200). **(G)** MMP-3 staining in endothelial cells at the lumen of an atherosclerotic aorta (original magnification x20). **(H)** Detail of (G). MMP-3 in endothelial cells (original magnification x 100). a=adventitia, ec=endothelial cells, i=intima, m=media, n=neomicrovessels.

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Chapter 8

Adenoviral delivery of a u-PA receptor-binding plasmin inhibitor and Green Fluorescent Protein: Inhibition of smooth muscle cell migration and visualisation of expression

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To be submitted

Summary

Approaches to the prevention of restenosis have so far shown little or no effect. Local release of inhibitors via gene therapy might, however, be more beneficial. Given the established role for the plasminogen activation system in cell migration and subsequent neointima formation, an approach to therapy is to overexpress an inhibitor of plasmin. Therefore, an adenoviral vector was constructed encoding the hybrid protein ATF.BPTI. This hybrid protein contains the active domain of bovine pancreatic trypsin inhibitor (BPTI), a potent inhibitor of plasmin, and the cell surface receptor binding domain of urokinase-type plasminogen activator (ATF), in order to target BPTI to the cell surface u-PA receptor. In addition, adenoviral vectors encoding BPTI and ATF alone were constructed. To identify the ATF.BPTI expressing cells, another adenoviral vector was constructed encoding ATF.BPTI and Green Fluorescent Protein (GFP) (Ad.ABIG). The latter construct was shown to be a valuable tool to monitor transfection efficiency and the behaviour of the transfected cells. Both the expression and functionality of the recombinant proteins was established in human vascular smooth muscle cells. We demonstrated that adenoviral gene transfer mediated expression of ATF.BPTI inhibited the migration of human saphenous vein SMC to a larger extent than expression of ATF or BPTI individually.

Introduction

Migration and proliferation of vascular smooth muscle cells (SMC) play a fundamental role in the development of intimal hyperplasia. Migration of cells within tissues requires controlled proteolytic degradation of the extracellular matrix (ECM) that encages them. Several proteolytic enzyme systems are thought to play a role in these processes, including the plasminogen activation (PA) system. The serine protease plasmin can not only directly degrade many components of the ECM but can also enhance pericellular proteolysis indirectly by activation of latent MMPs. The key component of the PA system is composed of the inactive proenzyme plasminogen that can be converted to its active derivative plasmin by two physiological plasminogen activators: urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). u-PA is thought to play a major role in local pericellular proteolysis, since it can bind to a specific cell surface receptor, the u-PAR. (1). A role for u-PA mediated plasminogen activation in migration has been reviewed for a variety of cells in general (2-4) and vascular SMC and arterial remodelling in particular (5). *In vitro* studies have described the inhibitory effects of plasminogen activator inhibitor-1 (PAI-1), aprotinin, soluble u-PAR, and antibodies to u-PA and t-PA on SMC migration. Furthermore, addition of exogenous u-PA, t-PA, or plasmin increases the migratory ability of SMC in *in*

vitro migration assays. (6,7). SMC isolated from u-PA or u-PAR deficient mice do not migrate in response to bFGF, while supplementation of cells from u-PA deficient animals with exogenous u-PA restores the migratory response (8). *In vivo* studies in balloon injured rat carotid arteries have shown an increase in u-PA, t-PA, PAI-1, u-PAR and overall plasmin activity at early times following arterial denudation (9,10). Furthermore, Carmeliet *et al* (11,12) have shown that in u-PA and plasminogen knockout mice neointima formation and neointimal cell accumulation were strongly reduced.

u-PA consists of three distinct domains: the first region from the N-terminus, referred to as the growth factor domain is followed by a region referred to as the kringle domain, which together are known as the aminoterminal fragment of u-PA (ATF). The third region contains the serine protease domain. Binding of u-PA to the u-PA receptor is mediated by its growth factor domain thereby retaining the proteolytic activity through its carboxyterminal region (13-15). Binding to its receptor not only restricts the activity of u-PA to the direct cell environment; it also brings u-PA in close contact to also cell-bound plasminogen, allowing the efficient activation of plasminogen to plasmin directly at the cell surface. Apart from its role in proteolysis, u-PA, or the receptor binding, non-proteolytic aminoterminal fragment, has been reported to have mitogenic effects on SMC (16) and various tumour cells (17-19).

Given the established role for the PA system in cell migration and subsequent neointima formation, an approach to therapy is to overexpress an inhibitor of plasmin. Therefore, an adenoviral vector was constructed encoding the active domain of bovine pancreatic trypsin inhibitor (BPTI), also known as aprotinin or Trasylol®, a potent inhibitor of plasmin. Since cell-associated plasmin is protected from inhibition by its natural inhibitors α_2 -antiplasmin and α_2-macroglobulin (20), an increased efficacy of BPTI is expected when it is brought to the cell surface. It has been reported that migrating cells overexpress the u-PA receptor (21), which is in close spatial proximity of the plasminogen receptor. Therefore we have constructed a second adenoviral vector encoding the hybrid protein ATF.BPTI, in order to target BPTI to the cell surface u-PA receptor. ATF, however, may achieve two additional effects apart from targetting BPTI to the u-PA receptor: 1) a decrease in proteolysis by making the u-PA receptor inaccessible for endogenous (pro)u-PA and thus reducing plasminogen activation, 2) an increase in proliferation due to the reported mitogenic effects of ATF. To study the role of these effects on SMC migration a third adenoviral vector was constructed encoding ATF.

To identify the ATF.BPTI expressing cells a fourth adenoviral vector was constructed encoding both ATF.BPTI and Green Fluorescent Protein (GFP), a protein originally discovered in the jellyfish *Aequorea victoria* and used widely as a non-invasive

autofluorescent marker for gene expression (22,23). Fusion of GFP to a gene of interest in an adenoviral vector would allow tracing of the transgene-producing cell. However, fusion of the two proteins may alter the tertiary structure and subsequently the functionality of the proteins. Therefore, to coexpress the GFP gene with another gene of interest, an internal ribosome entry site (IRES) sequence was placed between the ATF.BPTI and the GFP gene thereby creating Ad.ATF.BPTI.IRES.GFP (Ad.ABIG). This results in a construct with two independent translational units, allowing ATF.BPTI and GFP to be translated into two separate proteins from the same mRNA. Infection of cells with Ad.ABIG induces simultaneous expression of the secreted hybrid protein ATF.BPTI and GFP, which remains intracellular, providing us with a tool to monitor transfection efficiency and behaviour of the infected cells due to the visibility of the green fluorescent protein.

In this study we focus on the role of the PA system in SMC migration. We show that the cell surface binding plasmin inhibitor ATF.BPTI, expressed by SMC after adenoviral gene transfer, is a more potent inhibitor of saphenous vein SMC migration than ATF or BPTI individually.

Materials and Methods

Cell Culture

Human saphenous vein smooth muscle cells (SMC) were isolated from segments of saphenous vein obtained from patients undergoing coronary artery bypass grafting. Saphenous vein segments were kindly provided by Prof. H. Huysmans of the department of Thorax Surgery of the Leiden University Medical Centre, Leiden, The Netherlands according to the guidelines of the local ethical committee. After mechanical removal of endothelial cells, the adventitia was carefully stripped from the media. The media was then cut into small fragments and SMC were isolated via the explant method (24). Saphenous vein SMC were cultured in Dulbecco's modified Eagle medium supplemented with 2mM glutamine (DMEM, Gibco BRL, Paisley, Scotland), 5% (v/v) heat inactivated (30 min. 56°C) foetal calf serum (FCS), 5% (v/v) heat inactivated human serum, streptomycin (100 mg/mL) and penicillin (100 U/mL) (Biowhittaker, Verviers, Belgium) on gelatine (Merck, Darmstadt, Germany) coated dishes at 37 °C in a humidified 5% CO_2/95% air (v/v) atmosphere. The medium was renewed every 3 days and subcultures were established by trypsin/EDTA treatment of confluent cultures at a split ratio of 3. Human saphenous vein SMC were used for experiments between passage 2-6.

HER-911 cells (kindly provided by Dr. F. Fallaux (25), and CHO cells were cultured in DMEM supplemented with 10% (v/v) heat inactivated FCS (Life Technologies, Paisley, Scotland), and streptomycin (100 μg/mL)/penicillin (100 U/mL).

Construction of adenoviral vectors

The main features of the vectors described below are illustrated in Fig. 1.

pCMVATF.BPTI, an adenoviral shuttle vector encoding the ATF.BPTI hybrid protein was constructed by the following procedure. First, a vector containing the aminoterminal fragment of human u-PA, amino acids 1-138, referred to as ATF, was constructed by deleting the DNA sequences encoding amino acids

139 to 401 from a plasmid containing the full length u-PA using a polymerase chain reaction (PCR) with the oligonucleotides 5'CCCGGGCTTTTTCCATCTGCGCAGTC3' and 5'AGGGTCACCAAGGAAG-AGAATGGC3'. After amplification the DNA fragment was recircularised resulting in a plasmid encoding the ATF and 11 C-terminal amino acid residues of u-PA including the stop codon.
A DNA fragment encoding amino acid residues 36-93 of bovine pancreatic trypsin inhibitor (BPTI) was amplified by PCR on genomic DNA isolated from bovine aortic endothelial cells using the oligonucleotides 5'TCGCGACCTGACTTCTGCCTAGAGC3' covering nucleotides 2509 to 2533 of the BPTI gene according to the published sequence (GENBANK, BTBPTIG) and 5'GGTCACCCAGGGC-CCAATATTACCACC3' covering nucleotides 2677 to 2704 of the BPTI gene.
The amplified DNA fragments were then cloned into a pCRII cloning vector (Invitrogen, Carlsbad, CA, USA). A NruI/BsteII fragment of pCRIIBPTI was subsequently cloned into the SmaI/BsteII digested pCRIIATF vector to form the pCRII.ATF.BPTI vector. A HindIII/EcoRV fragment of pcrIIATF.BPTI was cloned in the EcoRV/HindIII digested adenoviral shuttle vector pCMV (fig. 1c). In order to construct pCMV.BPTI the sequences encoding aminoacids 2 to 137 were deleted from pCMV.ATF.BPTI using PCR with the oligonucleotides 5'TTCATTGCTGCCTTTGGTGTC3' and 5'CGACCTGACTTCTGCC-TAGAG3'. After amplification the DNA fragment was recircularised resulting in a pCMV.BPTI (fig. 1b).
To construct pCMVATF.BPTI.IRES.GFP, pBluescriptIRES.GFP (a kind gift from Dr.M. Heemskerk, Netherlands Cancer Institute) was digested with NotI, blunted with PWO DNA polymerase, and subsequently digested with EcoRV. The blunt-ended IRES.GFP fragment was cloned into the EcoRV site in pCMVATF.BPTI, which is located in between the ATF.BPTI gene and the polyA signal sequence as shown in figure 1d.
For the generation of the recombinant adenoviruses Ad.ATF, Ad.BPTI, Ad.ATF.BPTI and Ad.ABIG the pCMV constructs were co-transfected with pJM17 in HER 911 cells. Viral plaques were verified by PCR and restriction analysis. Viral stocks were prepared and frozen in aliquots with 15% (v/v) glycerol at -80°C.

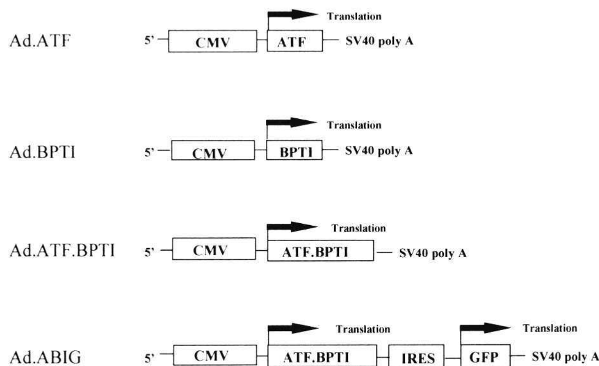

Figure 1. Expression cassettes of the adenoviral vectors used in this study.
The various protein coding sequences were placed under control of a CMV promoter. For more details on constructions see 'Materials and Methods'. The introduction of the Internal Ribosomal Entry Site (IRES) in the ABIG construct, allows the translation of both ATF.BPTI and Green Fluorescent Protein (GFP) from the same mRNA.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from human saphenous vein SMC by the Chomczynski procedure (26). 5 ug of total RNA was electrophoresed on a 1.2% (w/v) agarose-formaldehyde gel. The RNA integrity was confirmed by ethidium bromide staining. RNA was transferred to a nylon membrane filter (Amersham, Little Chalfont, England) and fixed by UV cross-linking. Filters were sequentially hybridised with [^{32}P] labelled DNA probes for ATF or BPTI.

Western Blotting

Samples of conditioned CHO medium were subjected to SDS-PAGE and subsequently blotted onto nitro-cellulose membrane as described previously (27). Recombinant proteins were visualised using rabbit anti-human u-PA polyclonal antibody (28) and the ECL Chemoluminescence Western Blot kit (Amersham, Buckinghamshire, UK) according to the manufacturers protocol.

ELISA

To measure recombinant protein expression in virus infected cells, ELISAs were set-up for ATF, BPTI, and ATF.BPTI. For the ATF ELISA the monoclonal antibody UK2.1, recognising the aminoterminal fragment of u-PA, was used as a catching antibody and a biotinylated rabbit polyclonal antibody directed against HMW u-PA was used as a detecting antibody (29). For the BPTI ELISA a rabbit polyclonal antibody raised against aprotinin (α-Trasylol) was used both as a catching and as a detecting antibody. In the ATF.BPTI ELISA, UK2.1 was used as a catching antibody and biotinylated α-aprotinin as a detecting antibody. Commercial standard u-PA (UKIDAN®, Serono, Aubonne, Switzerland) and aprotinin (Trasylol®, Bayer, Leverkusen, Germany) were used for reference values. To correct for possible variation in cell densities, the u-PA and aprotinin activities are expressed in ng and mU, respectively, per μg cell protein as determined by the BCA protein assay (Pierce). Since there is no standard for ATF.BPTI, values are given in OD 450. Endogenous u-PA production by the human saphenous vein SMC did not influence the ATF and ATF.BPTI measurements since recombinant protein production after infection was approximately 1000-fold higher.

Plasmin Activity Assay

Recombinant protein activity was analysed in a plasmin inhibition assay using the chromogenic substrate S2251 (Chromogenix, Mölndahl, Sweden). Diluted samples of the conditioned saphenous vein SMC media were incubated for 15 min at room temperature with 200 pmol/L plasmin. S2251 was added and after 24 h incubation at 37°C the A_{405} was measured to determine the conversion of the chromogenic substrate. As a control plasmin was incubated with buffer or with 1.0 KIU/mL aprotinin (Trasylol®, Bayer, Leverkusen, Germany).

Proliferation Assay

Incorporation of [^{3}H]-thymidine in DNA was determined as a measure of cell proliferation. Human saphenous vein SMC were seeded at 50% confluence in 24-well tissue culture plates and allowed to synchronise under serum-free conditions. After 24 h the medium was replaced by a concentration range of adenoviral dilutions and cells were infected for 24 h under serum free conditions. Cells were stimulated with DMEM supplemented with 10% (v/v) FCS 10% (v/v) human serum for 26 h at 37°C and labelled with 1μCi of [^{3}H]-thymidine/well (Amersham Pharmacia Biotech) for the last 4 h. Cells were then washed with ice-cold phosphate-buffered saline followed by ice-cold methanol and protein

was subsequently precipitated with 5% (v/v) trichloroacitic acid and dissolved in 0.3M NaOH. [^{3}H]-thymidine incorporation was measured using a scintillation counter.

Matrigel Invasion Assay

Invasion of human saphenous vein SMC was assayed using a Transwell system with Matrigel coated polycarbonate filters (6.5 mm diameter, 8 μm pore size, Costar, Cambridge, MA). Matrigel (Collaborative Biochemical Products, Bedford, MA) was layered onto the filter (14 μg/well) and allowed to dry at room temperature. The matrix was hydrated with DMEM before use. Cultured saphenous vein SMC ($4 * 10^4$ cells in 175 μl DMEM) were seeded after detachment by trysin/EDTA treatment in the upper chamber, and 900 μl DMEM was put in the lower compartment. Cells were allowed to attach to the matrix for 4 h before a concentration range of 25 μl adenovirus, or 25 μl diluted virus storage buffer as a control, were added to the upper compartments. After 16 h the medium of the upper chamber was replaced by 200 μl DMEM supplemented with 0.15 μmol/L plasminogen, and 900 μl DMEM supplemented with 0.15 μmol/L plasminogen and 10% FCS as chemoattractant replaced the medium in the lower chamber. As a control aprotinin (10-100 KIU/mL), was added to both upper and lower chambers. After 4 d of incubation at 37° C in 5% CO_2/95% air (v/v) the cells were fixed with 2.5% (v/v) glutaraldehyde. Matrigel and cells on the upper side of the filter were scraped off with a cotton swab, and cells on the lower side of the filter were stained with 2g/l crystalviolet. Invasion was determined by the % surface area covered by cells as measured by image analysis using a Nikon FXA microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software.

Analysis of GFP expression

Ad.ABIG infected SMC were lysed in 0.5% (v/v) TritonX-100, 30 min at 4° C. Fluorescence by infected cells was measured using a Cytofluor II (Perseptive Biosystems) with excitation at 485 nm and emission at 530 nm.

Detection of GFP expression in human saphenous vein SMC cultured on Matrigel coated Transwell filter membranes was achieved by carefully detaching the filter membranes from the inserts. After incubation in 6.8% (w/v) sucrose in PBS (15 min), the filters were embedded in Tissue tek. Frozen sections were cut at 5 μm and expression of Green Fluorescent Protein was monitored by fluorescence microscopy using a Nikon Microphot-FXA microscope with a FITC-filter Omega XF100.

Statistical Analysis

Data are presented as mean ± S.E.M. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference test. Statistical significance was accepted for $p<0.05$.

Results

Characterisation of recombinant ATF, BPTI, ATF.BPTI, and ABIG

Human saphenous vein SMC infected overnight with $1*10^8$ pfu/mL of Ad.ATF, Ad.BPTI, Ad.ATF.BPTI, or Ad.ABIG were subjected to Northern blot analysis in order to examine the mRNA expression of the various transgenes (Fig.2). The filter was sequentially hybridised

with an ATF probe and a BPTI probe and the presence and correct size of the two corresponding mRNAs was confirmed (1.25 kb and 1.0 kb respectively). Both probes also hybridised with a 1.5 kb band and a 2.9 kb band corresponding to ATF.BPTI mRNA and ABIG mRNA, respectively.

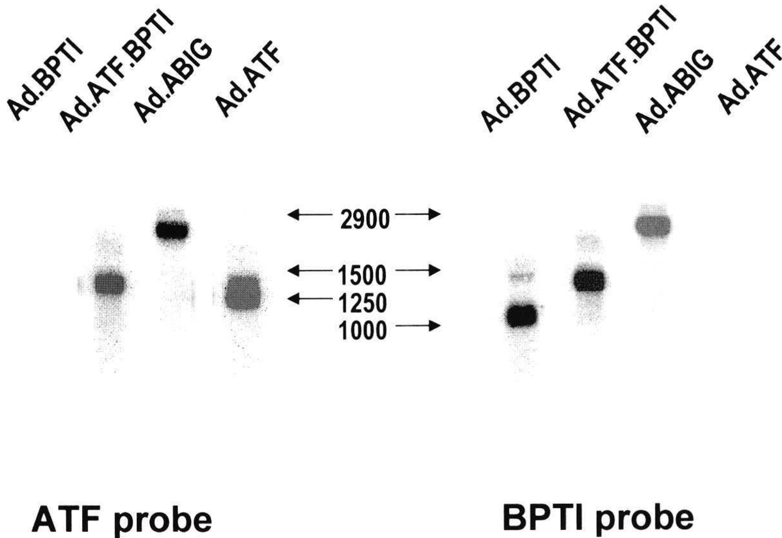

Figure 2. Northern blot analysis of Ad.ATF, Ad.BPTI, Ad.ATF.BPTI, and Ad.ABIG infected SMC.
Total cellular RNA was harvested from human saphenous vein SMC that were infected overnight with Ad.ATF, Ad.BPTI, Ad.ATF.BPTI, or Ad.ABIG. mRNA was analysed on Northern blots probed for ATF (**A**) or BPTI (**B**).

The production of the various proteins was visualised by Western blotting. Analysis was performed on conditioned medium from CHO cells infected with $1*10^8$ pfu/mL of the various adenoviral vectors (Fig.3). Using an antibody against human u-PA to detect the ATF domain, the presence and correct size of ATF (15 kDa) and ATF.BPTI (26 kDa) were confirmed. The same sized protein (26 kDa) was produced by Ad.ATF.BPTI and Ad.ABIG, although the mRNAs from the two constructs differ in size. This is due to the Internal Ribosome Entry Site (IRES) in the ABIG construct, which results in a separate translation of the ATF.BPTI and GFP proteins.

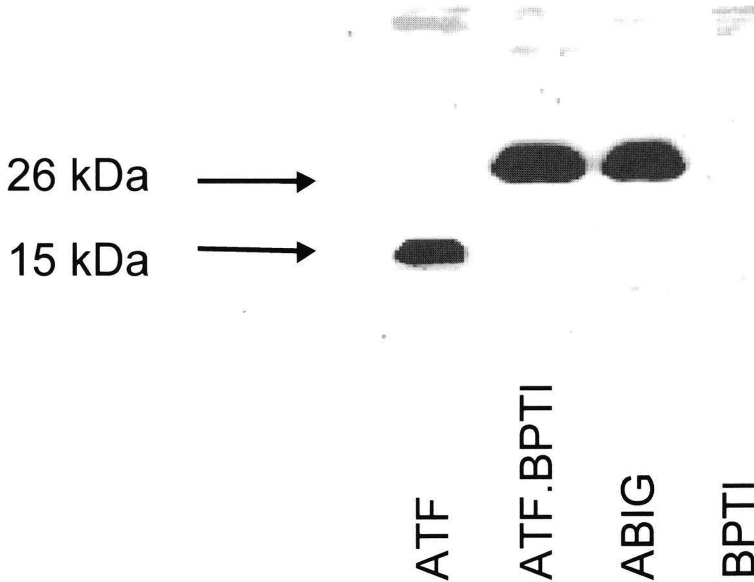

Figure 3. Western blot analysis of conditioned medium of CHO cells containing recombinant proteins.
Conditioned medium from CHO cells infected with Ad.ATF, Ad.ATF.BPTI, Ad.ABIG and Ad.BPTI were analysed by SDS gel electrophoresis and Western blotting. The blot was incubated with a rabbit anti-human u-PA polyclonal antibody recognising ATF and developed as described in 'Materials and Methods'.

Secretion of the recombinant proteins into the conditioned medium of virus infected human saphenous vein SMC was quantified by ELISAs for u-PA (recognising its amino terminal fragment), BPTI and the ATF.BPTI hybrid protein. The latter assay uses an anti-u-PA IgG as a catching antibody and an anti-BPTI IgG as a tagging antibody. The concentration of all three recombinant proteins increased dose-dependently in the medium with increasing adenovirus concentrations (not shown). In the ATF ELISA (fig.4a) a comparable production of ATF.BPTI was found in cells transfected with $1*10^8$ pfu/mL Ad.ATF.BPTI and Ad.ABIG (41.5 ± 7.9 and 38.4 ± 2.5 ng u-PA/μg cell protein, respectively) while ATF appeared to be produced more efficiently (61.1 ± 12.6 ng u-PA/μg cell protein). In the BPTI ELISA (fig.4b), SMC infected with $1*10^8$ pfu/mL Ad.BPTI, Ad.ATF.BPTI, or Ad.ABIG, gave comparable antigen levels (1.10 ± 0.20, 1.04 ± 0.09, and 0.99 ± 0.08 mKIU/μg cell protein respectively). In the ATF.BPTI specific ELISA only Ad.ATF.BPTI and Ad.ABIG infected SMC (10^8 pfu/mL) gave a strong and comparable signal (fig.4c). Because the ABIG construct is transcribed into one mRNA from which two separate proteins are translated, a linear

correlation between the production of ATF.BPTI and GFP is expected. Figure 4d shows that a dose-dependent and linear increase in both ATF.BPTI antigen and GFP-reflecting fluorescence intensity was observed in SMC infected with a range of concentrations of Ad.ABIG.

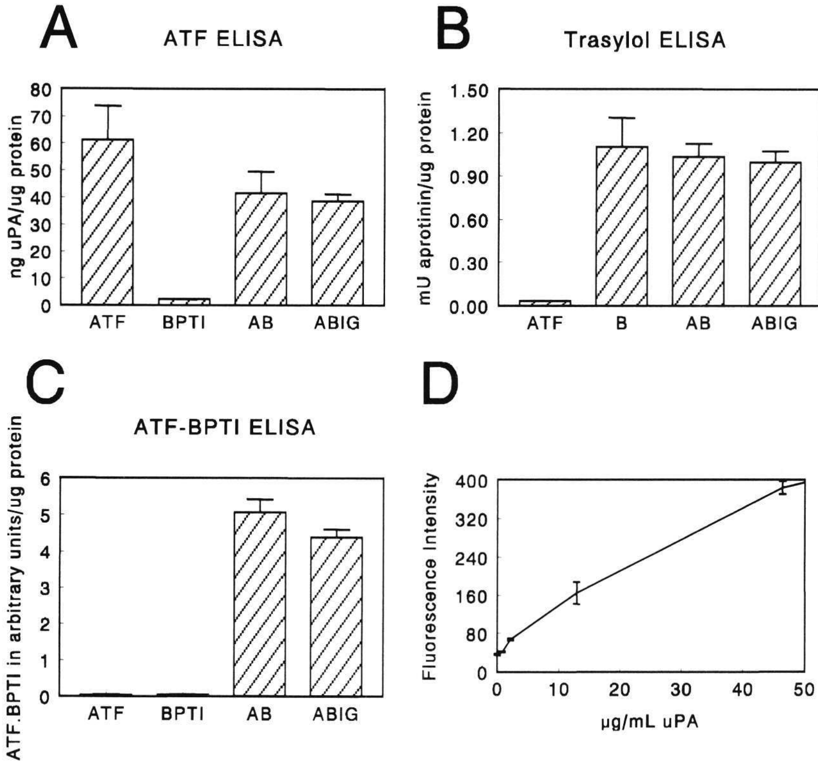

Figure 4. Quantitative comparison of recombinant protein production.
Conditioned medium of human saphenous vein SMC infected with Ad.ATF, Ad.BPTI, Ad.ATF.BPTI, and Ad.ABIG was tested in the ATF ELISA (**A**), Aprotinin ELISA (**B**), and the specific ATF.BPTI ELISA (**C**) as described under 'Materials and Methods'. Cell extracts of SMC infected with various concentrations of Ad.ABIG were measured by fluorimetry and the corresponding conditioned media in the ATF ELISA (**D**).

Inhibition of plasmin activity

Plasmin inhibitory activity was measured to assess the functionality of the secreted recombinant proteins. In a plasmin inhibition assay, diluted samples of conditioned medium

from human saphenous vein SMC infected with $1*10^7$ pfu/mL of the various adenoviral vectors were analysed. Whereas medium of Ad.ATF transfected cells had no inhibitory effect on plasmin activity, medium of Ad.ATF.BPTI, Ad.ABIG, and Ad.BPTI transfected cells was capable of inhibiting 200 pmol/L plasmin by more than 90% up to a 1:16 dilution. This is comparable to the effect of 1 KIU/mL of aprotinin (Fig.5). A significant inhibition of plasmin activity was observed up to a dilution of 1:256.

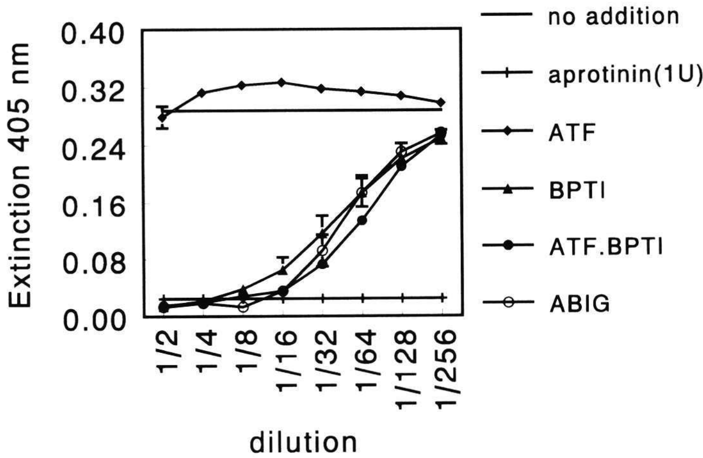

Figure 5. Plasmin inhibition assay.
Diluted samples of conditioned medium from human saphenous vein SMC infected with Ad.ATF, Ad.BPTI, Ad.ATF.BPTI, or Ad.ABIG were analysed for plasmin inhibitory activity at the indicted dilutiond. Complete inhibition of plasmin activity was established by addition of 1 KIU/mL aprotinin. Details are described in 'Materials and Methods'.

To demonstrate that ATF.BPTI is able to inhibit plasmin activity at the cell surface, mouse LB6 cells expressing the human u-PA receptor (uPAR), as well as control LB-6 cells, were incubated for 1 hr with ATF.BPTI-containing CHO medium. After extensive washing, cell extracts were prepared and the plasmin inhibitory capacity was determined as described in Materials and Methods. Plasmin inhibition (93.2 ± 2.1%) could only be detected in extracts of uPAR expressing cells but not in the control LB6 cells (Table I). Inhibition of plasmin activity at the surface of human saphenous vein SMC was achieved in a similar way by incubating SMC with the ATF.BPTI-containing CHO cell culture medium and resulted in a plasmin activity inhibition of 85.2 ± 3.9%. Binding of u-PA to its receptor uPAR is reversible and

receptor-bound u-PA can be removed by a mild acid treatment. This feature was used to further demonstrate that functional ATF.BPTI was bound to the uPAR on the cells. After incubation of the cells with ATF.BPTI medium and extensive washing, part of the cells underwent a mild acid treatment (2 min incubation with pH 3.0 glycine buffer) before cell lysates were prepared. No plasmin inhibitory activity could be detected in the lysates of the acid treated cells. Incubation of SMC with BPTI-containing CHO conditioned medium was not capable of inhibiting plasmin activity before or after the acid treatment. These results indicate that ATF successfully anchors BPTI to the u-PAR where it can inhibit plasmin activity at the cell-surface.

	Before acid treatment	After acid treatment
	inhibition plasmin activity by ATF.BPTI	
control LB 6	0%	0%
uPAR LB6	93.2 ± 2.1%	0%
SMC	85.2 ± 3.9%	0%
	Inhibition plasmin activity by BPTI	
SMC	0%	0%

Table I Inhibition of plasmin activity at the cell-surface.

Efficiency of adenoviral transfection of human saphenous vein SMC

To determine the adenovirus concentration for optimal transfection, $4*10^4$ human saphenous vein SMC were seeded onto 48-wells plates and infected with a range of concentrations Ad.ABIG for 16 hours. Expression of ABIG was determined by measuring relative Green Fluorescent Protein fluorescence in cell extracts after 48 hours. A dose dependent increase of fluorescence with adenovirus concentration was found up to a maximum for $6*10^8$ pfu/mL (Fig. 6). Infection with vector concentrations > $1*10^9$ pfu/mL resulted in a cytotoxic effect as shown by a decrease in relative fluorescence and by changes in cell morphology (not shown). To be sure that no cytotoxic side effects would occur, a maximum adenovirus concentration of $1*10^8$ pfu/mL was chosen for further experiments.

Effect of ATF and ATF.BPTI expression on human saphenous vein SMC proliferation

Possible mitogenic effects in response to expression of increasing concentrations of ATF on human saphenous vein SMC were determined by measuring [^{3}H]-thymidine incorporation of Ad.ATF infected cells in comparison with Ad.BPTI, AdATF.BPTI, and control vector (Ad.LacZ) infected cells (Fig.7). Although [^{3}H]-thymidine incorporation was slightly

decreased by adenoviral infection *per se*, no significant differences in incorporation were detected between the various viral constructs. This indicates that under the conditions used in our experiments, expression of ATF or ATF.BPTI in SMC has no effect on their proliferation.

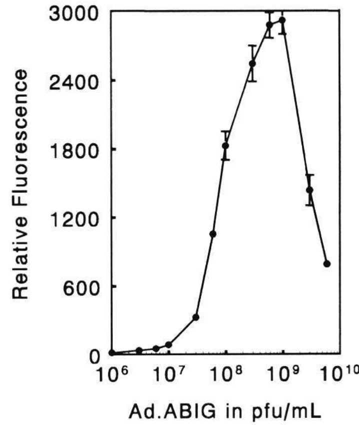

Figure 6. GFP expression as measured by relative fluorescence
Cell extracts of Ad.ABIG infected SMC were analysed for GFP expression by fluorescence measurement. Viral concentrations used for infection are indicated, Details can be found in 'Materials and Methods'.

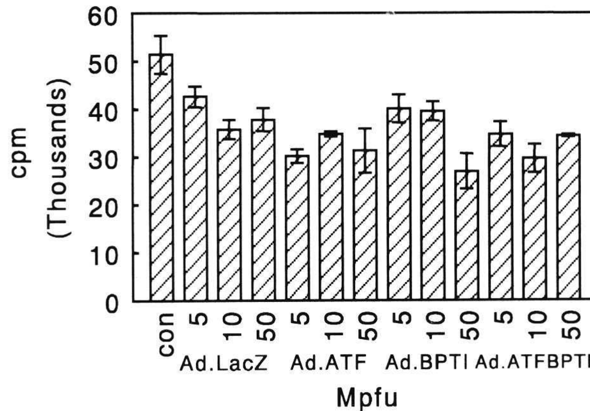

Figure 7. Proliferation assay
The proliferation of human saphenous vein SMC infected with Ad.ATF, Ad.BPTI, Ad.ATF.BPTI, or Ad.LacZ as a control vector was determined by measuring [^{3}H]-thymidine incorporation. As an additional control the proliferation of non-infected SMC was measured (con). Results are expressed in counts per minute (cpm) ± S E M.

Effect of ATF and ATF.BPTI expression on human saphenous vein SMC migration
SMC migration is dependent on degradation of the extracellular matrix by cellular proteases. Therefore, we studied the effects of inhibiting plasmin activation by adenoviral infection of saphenous vein SMC with Ad.ATF, Ad.BPTI, Ad.ATF.BPTI and with Ad.LacZ as a control, in a Transwell filter assay. As seen in Fig 8, human saphenous vein SMC are able to migrate across the Matrigel coated porous filter under control conditions. Addition of aprotinin reduces migration by 52% at a concentration of 100 KIU/mL.

Control LacZ coding adenovirus (Ad.LacZ) had hardly any effect on migration up to $1*10^7$ pfu/mL. Ad.ATF.BPTI inhibited migration between 33 and 55% of LacZ control in this concentration range, whereas Ad.ATF and Ad.BPTI had no significant effects on migration. At higher virus concentrations Ad.LacZ also inhibited migration significantly, although the inhibiting effect of Ad.ATF.BPTI was always significantly higher (Fig. 8).

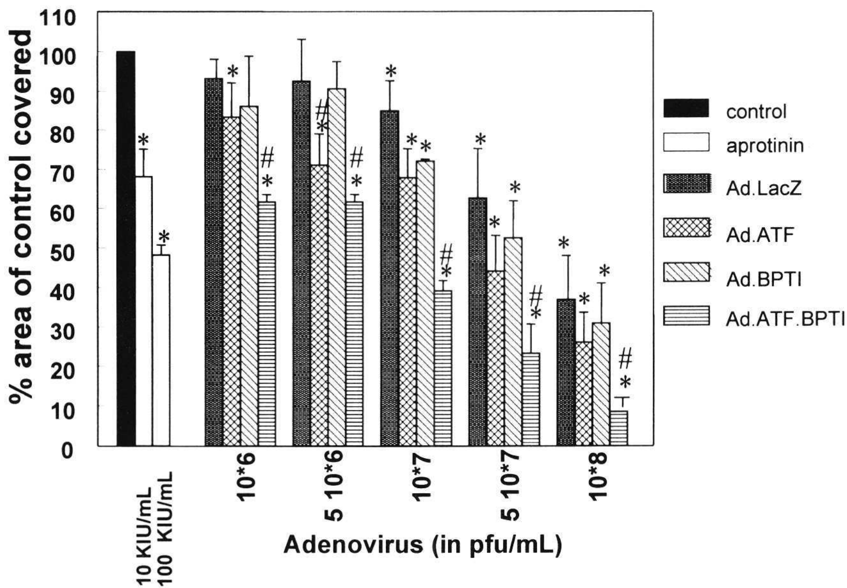

Figure 8. Migration assay
The migration of human saphenous vein SMC infected with Ad.ATF, Ad.BPTI, Ad.ATF.BPTI, or Ad.LacZ as a control vector was determined by quantification of the number of cells that migrated through the Matrigel-coated Transwell filter. The results are presented as percentage of control ± SEM. * $P<0.05$ compared to control (no virus), # $P<0.05$ compared to the corresponding Ad.LacZ virus concentration.

Monitoring of Ad.ABIG infected smooth muscle cells.

Infection with Ad.ABIG gives insight into the behaviour of the infected cells due to the visibility of the Green Fluorescent Protein. As shown in Fig. 9a, infection with $5*10^7$ pfu/mL gives rise to more than 95% infection of SMC. Subsequent cleaning of the upper compartment removes all GFP expressing cells (Fig.9b), indicating that the cells infected with GFP did not migrate through the Matrigel layer and the SMC that did migrate across the filter did not express GFP and consequently also no ATF.BPTI. A crossection of a Matrigel coated Transwell filter, 4 days after infection, also shows fluorescent cells only in the upper compartment but not on the underside of the filter (Fig.9c).

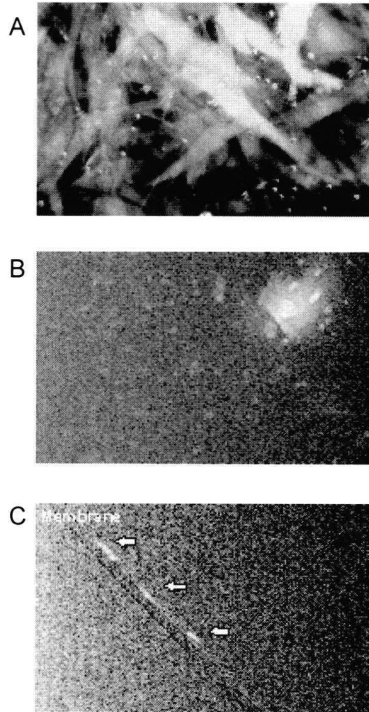

Figure 9. Effect of GFP expression on SMC migration

SMC were placed on Matrigel coated filters and infected with Ad.ABIG. Four days after infection migration of cells was analysed by fluorescence microscopy, showing cells on both sides of the filter (**A**). After removing cells from the upper compartment, cells migrated through the filter were photographed (**B**). The filter was removed from its holder, embedded in Tissue tek and frozen. Sections of 5µm were analysed for GFP fluorescence (**C**).

Discussion

Pharmacological approaches to the prevention of restenosis have so far shown little or no effect or provide conflicting data. (30,31) Therefore research has been directed to the development of novel strategies such as gene therapy in the hope that local release of inhibitors may be more beneficial. In animal studies both inhibition of proliferation and inhibition of migration have been shown effective in reducing intimal hyperplasia (32).

In this study we studied a gene transfer approach to inhibition of SMC migration, aimed at inhibition of pericellular plasmin activity. A novel adenoviral construct was devised, encoding the hybrid protein ATF.BPTI, which contains the active domain of bovine pancreatic trypsin inhibitor (BPTI), a potent inhibitor of plasmin, and ATF, the receptor binding domain of u-PA, in order to target BPTI to the cell surface u-PA receptor. The effects of infection of SMC with Ad.ATF.BPTI on plasmin inhibition and cell migration were compared with adenoviral constructs encoding BPTI and ATF alone. To identify the ATF.BPTI expressing cells, an adenoviral vector was constructed encoding ATF.BPTI fused to Green Fluorescent Protein (GFP) (Ad.ABIG).

Infection of saphenous vein SMC with various concentrations of the adenoviral constructs led to a dose-dependent production of the recombinant proteins as determined by ELISA. Both BPTI containing constructs; BPTI and ATF.BPTI, were produced in similar amounts based on a aprotinin ELISA. In the u-PA ELISA however, ATF appears to be produced more efficiently than ATF.BPTI. This difference in transgene expression was in agreement with mRNA levels as observed by the Northern blot analysis.

Conditioned medium from human saphenous vein SMC infected with Ad.BPTI or Ad.ATF.BPTI, was capable of inhibiting plasmin activity. Whereas medium from cells infected with Ad.ATF had no inhibitory effect. Furthermore, it was found that lysates of SMC incubated with ATF.BPTI conditioned medium showed a profound inhibition of plasmin activity whereas cell lysates incubated with BPTI conditioned medium had no inhibitory effect. This indicates that fusion of BPTI to ATF directs its plasmin inhibiting capacity to the cell-surface, as expected.

We tested the effects of adenoviral gene transfer with the various constructs in a Transwell filter migration assay. Infection of SMC with Ad.ATF.BPTI resulted in a strong inhibition of SMC migration varying between 32 and 78% of LacZ control. The effects observed for Ad.ATF and Ad.BPTI were similar as the effects for Ad.LacZ. At virus concentrations higher than $1*10^7$ pfu/mL cytotoxic effects of viral transfection *per se* did occur; although also at these concentrations Ad.ATF.BPTI had more inhibiting effect than any of the other viral constructs.

We show that ATF.BPTI linked to GFP with an IRES sequence is expressed in a proportional relationship, such that the magnitude of expression of GFP reflects the magnitude of expression of ATF.BPTI. The bicistronic co-expression of a therapeutic and an autofluorescent marker gene provides us therefore with a tool to monitor gene transfer and the subsequent behaviour of the transfected cells. In a migration assay with Ad.ABIG transfected cells, it was shown that fluorescent cells, expressing GFP and ATF.BPTI, did not migrate. Only non-fluorescent cells were observed at the underside of the filter, again indicating that ATF.BPTI efficiently interferes with *in vitro* cell migration.

Interference with the plasmin system leads to partial inhibition of SMC migration as observed in this study and in previous studies (6-8). This suggests that other mechanisms might also be involved. Recently several investigators have shown that local overexpression of inhibitors of the matrix metalloproteinase system might also be effective to inhibit SMC migration (33-36). Various studies have claimed that occupancy of u-PAR by u-PA not only influences proteolysis, but also initiates intracellular signalling events promoting cell migration (37,38) and proliferation (17,19,42). Addition of ATF has been reported to increase migration of melanoma cells and bovine endothelial cells *in vitro* (39). However, Li *et al.* (40) observed that adenoviral delivery of ATF to various tumour-types in murine models led to *inhibition* of metastasis. Furthermore, addition of soluble u-PA receptor or a receptor blocking antibody to an *in vitro* angiogenesis model was shown to prevent migration and subsequent tube-formation by microvascular endothelial cells in a fibrin matrix (41).

Intracellular signalling mediated via the u-PAR has also been implicated in cell proliferation. *In vitro* studies have described the mitogenic effects of HMW-u-PA and ATF in various cell types (17,19,42). To assess if effects on proliferation may have influenced the results obtained in the invasion assay, a tritiated thymidine incorporation assay was performed. Thymidine incorporation by Ad.ATF infected HSVSMC, however, did not significantly differ from Ad.BPTI, Ad.ATF.BPTI, or control virus infected SMC. The inhibition of SMC migration by Ad.ATF.BPTI is not caused by an inhibition of proliferation or signalling, but appears completely due to inhibition of local plasmin activity.

In conclusion, our experiments show that local expression of the novel hybrid protein ATF.BPTI leads to profound inhibition of SMC migration. Expression of ATF and BPTI has no effect. This indicates that targetting BPTI action to the cell-surface leads to a substantial increase in effectivity. Local expression of this hybrid protein by adenoviral gene transfer might be a promising approach for inhibiting SMC migration and consequently restenosis.

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Chapter 9

Summary and Discussion

In the processes of atherosclerosis and restenosis, endothelial cell and smooth muscle cell proliferation and migration occur. As a reaction to injury to the vessel-wall, smooth muscle cells migrate from the media to the intima and start to proliferate (1-3). The resulting thickening of the intima can cause a narrowing of the lumen of the vessel-wall. Such a narrowing can cause thrombosis and the vessel can become partly or even completely occluded, with often dangerous or fatal consequences. Migration of smooth muscle cells is thought to be one of the most important processes in vessel-wall thickening. It is known from other processes in which cell-migration is involved, like for example in wound healing, angiogenesis, and tumour cell invasion, that extracellular proteolysis plays an important role (4-8). Proteolytic enzymes dissociate the cells from the extracellular matrix in which they are encaged, enabling cells to migrate. At least two types of proteolytic enzyme systems are involved in cell-migration: the plasminogen activation system and the matrix metalloproteinases.

At the time the studies described in this thesis were initiated, not much was known about the role of proteolytic enzymes in the migration of vascular cells. In the research described in this thesis, we used *in vitro* model-systems to study the migration of human smooth muscle cells and endothelial cells. In these models we examined which enzyme systems are involved in vascular cell migration. Furthermore, we examined if the inhibition of these enzymes via gene-therapy might be a successful intervention for restenosis and atherosclerosis.

Involvement of the plasminogen activation system in the migration and invasion of human smooth muscle cells.

We made use of two in vitro model-systems to study human smooth muscle cell migration: a model to study lateral migration of smooth muscle cells over an extracellular matrix synthesised by the cells themselves, and a model for invasive migration of smooth muscle cells through an extracellular matrix. In the first model a cell-free "wound" is created by stripping smooth muscle cells from their extracellular matrix with a Millipore filter strip. The repopulation of the stripped area is followed microscopically in time. In the second model smooth muscle cells are cultured on a commercial extracellular matrix (Matrigel®) that is coated on Transwell® filters with micropores. After migrating through the pores, the appearance of cells on the underside of the filters can be determined.

We show that both the addition of t-PA or of u-PA leads to an increase in human umbilical vein smooth muscle cell (HUVSMC) migration in the lateral wound essay (chapter **2**) and in invasion through Matrigel® (chapter **4**). The formation of plasmin from plasminogen is

essential in this process, because the addition of an inhibitor of plasmin (aprotinin) can inhibit the stimulatory effects of both plasminogen activators and addition of plasmin itself also stimulates HUVSMC migration.

Several components of the plasminogen activation system (u-PA, t-PA, PAI-1 and u-PAR) are expressed by human smooth muscle cells (chapters **3-5**, **8**). Both in lateral migration and invasion, the addition of specific blocking antibodies directed against u-PA or t-PA inhibited smooth muscle cell migration and invasion. This inhibition usually was not more than 50%, suggesting that next to the plasminogen activation system, also other systems are involved in smooth muscle cell migration.

We examined the involvement of the urokinase receptor with a soluble form of u-PAR, lacking the GPI-anchor, but still able to bind to u-PA. When sol-u-PAR was added, HUVSMC migration was reduced (chapter **3**), suggesting that the binding of u-PA to its cell-surface receptor is stimulating migration. The addition of LMW-u-PA (which lacks ATF, the u-PAR binding domain of u-PA) or ATF (which lacks the proteolytic domain of u-PA) did not stimulate lateral HUVSMC migration, although the activity of LMW-u-PA would have been able to convert plasminogen into plasmin, thereby facilitating migration. These results suggest that for stimulation of lateral migration by u-PA both activity *and* binding to the cell-surface receptor are necessary. Furthermore, the receptor associated protein (RAP), an antagonist of receptors from the low-density lipoprotein (LDL) family, was able to inhibit smooth muscle cell migration and invasion. This suggests that these types of receptors play a role in smooth muscle cell migration via their effect on cell-surface proteolysis via the uptake of plasminogen activator/inhibitor complexes. Together these results indicate that plasmin formation at the cell-surface might be the critical factor in the migration process.

Recently, Carmeliet and coworkers have generated knock-out mice that are deficient in components of the fibrinolytic system (9-14). All these knock-out mice are viable, and able to reproduce. To study the restenotic process in these mice experimental models were used that were either based on the application of an electric current or on an intraluminal guidewire. In both models the rate and degree of neointima formation and neointimal cell accumulation after injury were similar in the wild-type, t-PA deficient and u-PAR deficient arteries (15,16). However, neointima formation in PAI-1 deficient arteries occurred earlier after injury (17). In contrast, both the degree and the rate of arterial neointima formation in u-PA deficient, plasminogen deficient, and combined u-PA:t-PA deficient arteries were significantly reduced (15,18). u-PA and PAI-1 expression were markedly upregulated during vascular wound healing (15,17). Proliferation of smooth muscle cells was not significantly different between

the genotypes, suggesting that an impaired migration of smooth muscle cells is the cause of reduced neointima formation in plasminogen and u-PA deficient mice. That u-PAR deficient arteries developed a similar degree of neointima formation as did wild-type arteries was not due to a lack of u-PAR expression in wild-type arteries (16). u-PA was present on the cell-surface of wild-type smooth muscle cells, and accumulated in the pericellular environment around u-PAR deficient smooth muscle cells. These results in mice suggested that binding of u-PA to u-PAR is not required to provide sufficient pericellular u-PA mediated plasmin proteolysis to allow smooth muscle cell migration into the vascular wound. These results obtained in these knock-out mice are partly different from the results we obtained in our human *in vitro* cell migration models, because we found that next to u-PA as in in the mice, also t-PA and u-PAR are involved in smooth muscle cell migration. An explanation for these results could be species differences and/or the use of different migration model-systems. In our *in vitro* models u-PAR participates in the regulation of smooth muscle cell migration, but it does not seem to be essential. For an efficient migration to occur, plasmin formation is necessary. In our *in vitro* models it does not matter if this plasmin is formed from plasminogen by u-PA or by t-PA.

Differences in invasion behaviour of smooth muscle cells, isolated from different vessels.

It is known that smooth muscle cells are heterogeneous and can express a range of phenotypes, ranging from the contractile to the synthetic phenotype (19,20). It is suggested that there are differences in smooth muscle cell phenotype between vessels (21,22). This might explain the variability of vessels to develop intimal thickenings after vascular injury. Because the plasminogen activation system was shown to be important in one of the key processes of intima formation (smooth muscle cell migration), we studied the expression of the plasminogen activation system and its role in migration of smooth muscle cells isolated from several human vessels.

In chapter **4** we determined that smooth muscle cells, isolated from different human blood-vessels (umbilical vein, saphenous vein and mammary artery), have differences in their *in vitro* migratory behaviour through Matrigel®. These differences could be partly explained by differences in the levels of t-PA and PAI-1 produced by these cells, while the expression of u-PA did not differ much between the different smooth muscle cell-types studied. Our results suggest that not only phenotypic differences exist between smooth muscle cells from the intima and media (19,20), but also between smooth muscle cells from different human

vessels. These findings might explain (in part) the variability of vessels to develop vascular disease.

Regulation of plasminogen activation by cell-density

In the lateral migration model, migration of smooth muscle cells starts after the removal of cells. A possible trigger for migration could be the (local) cell-density. We show in chapter **5** that the cell-density of smooth muscle cells in culture can influence the levels of t-PA and PAI-1 in the culture medium, but not the levels of u-PA. For t-PA and PAI-1 there was a strong negative correlation between the amounts of t-PA and PAI-1 accumulating in the culture medium and smooth muscle cell density. In chapter **3** we showed that the addition of RAP (receptor associated protein, an antagonist for binding of ligands to members of the LDL-receptor family) could inhibit smooth muscle cell migration and invasion. RAP and other members of the LDL-receptor family can play an important role in the clearance of plasminogen activator:PAI-1 complexes and of t-PA from the local environment, thereby regulating the proteolytic activity on the cell-surface. We determined that the effect of cell-density on the accumulation of t-PA and PAI-1 could be explained for a large part by differences in the uptake of t-PA and PAI-1 via receptors of the LDL-receptor family, most likely via the VLDL-receptor. In this way cell-density could indirectly modulate migration and invasion of SMC. Whether this mechanism also plays a role in the regulation of migration *in vivo* remains to be determined.

Comparison of migratory/invasion behaviour of endothelial cells and smooth muscle cells

In chapter **6**, we found striking differences in the migration of endothelial cells and smooth muscle cells. As discussed, the plasminogen activation system is involved in lateral migration of smooth muscle cells. Interfering in this system, however, did not show any effects on the lateral migration of endothelial cells. Endothelial cells, however, do utilise the plasminogen activation system for their invasive growth into fibrin-matrices. As shown by Koolwijk et al. (7), receptor bound u-PA mediated plasminogen activation is involved in the angiogenic invasion of fibrin matrices whereas t-PA mediated plasminogen activation is not involved. Smooth muscle cells have such a pronounced plasminogen activator activity (mainly t-PA) that they can lyse the fibrin matrix totally and therefor cannot use the fibrin as a supportive

matrix. In contrast, smooth muscle cells do utilise the plasminogen activation system when migrating over or through an extracellular matrix.
Together, these observations suggest that the role of the plasminogen activation system in vascular remodelling is both dependent on the cell-type (smooth muscle cells versus endothelial cells), but also on the type of vascular remodelling and migration (lateral migration versus invasion).

Proteolytic enzyme-systems in human blood-vessels

In chapter **7** we used immunohistochemical analysis to examine the expression of components of the plasminogen activation system and the matrix metalloproteinases in healthy, narrowed, and atherosclerotic blood-vessels.
While MMP-9 staining was increased in atherosclerotic arteries, staining of TIMP-1 was decreased. There were no significant differences in u-PA staining in healthy and atherosclerotic vessels, while t-PA was staining less in the media of atherosclerotic vessels and PAI-1 was elevated in the intima of atherosclerotic vessels. Noticeable was that the urokinase receptor was elevated in the intima of thickened vessels and lowered in the intima of atherosclerotic vessels. Our results suggest that the ultimate MMP-activity might be increased in atherosclerotic arteries, while the plasminogen activator activity might be decreased. This suggests that the matrix metalloproteinase system and the plasminogen activator system may play different roles in the process of atherosclerosis. The general conclusion is that the plasminogen activation system and the matrix metalloproteinases are regulated, locally and in time (phase of atherosclerosis/intima thickening), in agreement with a possible role in cell-migration processes in the vessel wall.

Gene-transfer aimed at inhibition of local proteolysis as a possible therapeutic intervention

Local proteolysis and the plasminogen activation system in particular, play an important role in cell-migration processes in the vessel-wall. In chapter **8** we started to investigate a possible intervention by making a hybrid protein with a u-PA receptor-binding domain for localisation to the cell-surface (ATF-domain) and a plasmin-inhibiting domain (bovine pancreas trypsin inhibitor). Through adenoviral gene-transfer this protein could be locally produced by cultured saphenous vein smooth muscle cells. In our Matrigel® invasion assay smooth muscle cell invasion could dose-dependently be inhibited by adenoviral gene-transfer.

General conclusions

From our investigations *in vitro* and that of others *in vivo* it is clear that the plasminogen activation system plays an important role in the migration and invasion of smooth muscle cells. Interfering with this system is able to inhibit in vitro migration and invasion. For efficient migration it is necessary that the amount and activity of plasminogen activators is tightly regulated. In this thesis we show that the plasminogen activation system is regulated by the density of smooth muscle cells and that there are also differences in plasminogen activator expression in smooth muscle cells between human vessels.

The first experiments with a gene-transfer approach aimed at inhibition of cell-bound proteolysis appear to be able to inhibit smooth muscle cell migration. Preliminary results of experiments in animal models suggest that this approach might prove valuable in preventing restenosis after coronary angioplasty.

In summary, the results described in this thesis provide more insight in the involvement of the plasminogen activation system in the migration in smooth muscle cells. This insight can be used to devise new methods for the inhibition of restenosis.

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Chapter 10

Nederlandse samenvatting

Atherosclerose (aderverkalking) is een van de belangrijkste doodsoorzaken in de Westerse wereld. Atherosclerose ontstaat als het gevolg van beschadigingen van de vaatwand die o.a. kunnen worden veroorzaakt door roken, een hoge bloeddruk en een hoog cholesterolgehalte. Als reparatie-reactie op beschadigingen van de vaatwand gaan de cellen die het grootste deel van de vaatwand uitmaken (de gladdespiercellen) zich delen en ze migreren vanuit het midden van de vaatwand (media) naar de binnenwand van de vaatwand (de intima). Ook stapeling van cholesterol en vorming van bindweefsel en cellen draagt bij aan de vernauwing. Wanneer een vat vernauwd raakt kan het bloed slechter door het vat stromen. In de vernauwing kunnen zich bij een beschading ook makkelijk bloedstolsels vormen. Dit kan uiteindelijk zelfs aanleiding geven tot een volledige afsluiting van het vat waardoor de achterliggende weefsels niet voldoende zuurstof meer kunnen krijgen en afsterven. Men spreekt dan van een infarct.
Om een (gedeeltelijk) afgesloten bloedvat weer ruimer te maken zijn er verschillende technieken ontwikkeld. De bekendste hiervan zijn de bypass (hierbij wordt een omleiding gemaakt) en het dotteren (het oprekken van een atherosclerotisch vat met een ballonnetje). Omdat er bij deze technieken vaak ook vaatwandbeschadigingen optreden vind er in 30-40% van de behandelingen na verloop van tijd een nieuwe verdikking van de vaatwand plaats. Dit proces wordt restenose genoemd.
Er wordt veel onderzoek gedaan naar de mechanismen en de regulatie van bovengenoemde gladdespiercel migratie. Uit onderzoek naar andere processen waarbij celmigratie een rol speelt blijkt dat de afbraak van de matrix die rond de cellen zit een belangrijke rol speelt. Proteolytische eiwitafbrekende) enzymen maken de cellen los van hun omgeving waardoor deze cellen in staat zijn om verder te migreren. Er zijn tenminste twee typen van proteolytische enzymsystemen bekend die hierbij een rol spelen: het plasminogeen activatie systeem en de matrix metalloproteinases.
In dit proefschrift hebben we de rol van deze enzymsystemen op het migratie proces van humane gladdespiercellen bestudeerd.

Betrokkenheid van het plasminogen activatie systeem bij de migratie en invasie van gladdespiercellen.

In onze studies hebben we gebruik gemaakt van humane gladdespiercellen, die werden vrijgemaakt uit kleine stukjes vaten die bij een operatie beschikbaar kwamen. Deze cellen werden gekweekt in plastic kweekschaaltjes. Om de migratie van gladdespiercellen te bestuderen maakten we gebruik van twee modelsystemen. In het eerste model lieten we kweekschaaltjes vol groeien met gladdespiercellen. Hierna werd er een smal strookje filterpapier op de cellen gedrukt en weer weggehaald. Op de plek waar het strookje had

gezeten onstond een leeg gebied zonder cellen, een zogenaamde "wond". Als reactie hierop migreerden de gladdespiercellen in het lege gebied om dat te "repareren". Met behulp van een microscoop konden we deze migratie in de tijd volgen.
In het tweede model werden gladdespiercellen op een matrixlaag van Matrigel gekweekt. Deze matrixlaag lag op een filter met hele kleine gaatjes waar de gladdespiercellen net doorheen konden kruipen. De gladdespiercellen werden gestimuleerd om door de matrixlaag en de gaatjes van het filter heen te migreren. Met behulp van een microscoop kon het aantal cellen dat uiteindelijk door de gaatjes was gekropen naar de onderkant van het filter worden geteld.
De betrokkenheid van plasminogeenactivatoren werd bestudeerd door remmende antilichamen tegen deze proteolytische enzymen aan onze twee migratie-modellen toe te voegen. In hoofdstuk 3 konden we aantonen dat wanneer de activiteit van zowel het urokinase-type plasminogeen activator (u-PA) als van het tissue-type plasminogeen activator (t-PA) werd geremd met deze antilichamen ook de migratie van gladdespiercellen werd geremd. In hoofdstuk 2 en 4 lieten we zien dat het toevoegen van u-PA of t-PA in onze model-systemen de migratie van gladdespiercellen stimuleert. Wanneer we het eindprodukt van het plasminogeen activatie-systeem remden (het proteolytische enzym plasmine), werd ook de migratie geremd. Verder konden we aantonen dat een aantal receptoren die betrokken zijn bij het plasminogeen activatie systeem ook betrokken zijn bij gladdespiercel migratie nl.: een specifieke cel-oppervlakte receptor voor u-PA (u-PAR) en receptoren (LRP en de VLDL-receptor) die betrokken zijn bij o.a. de opname door cellen van complexen bestaande uit plasminogeen activatoren en hun specifieke remmer PAI.

Verschillen in de migratie van gladdespiercellen uit verschillende menselijke vaten

In hoofstuk 4 hebben we de migratie van gladdespiercellen door matrix heen bestudeerd. Deze gladdespiercellen waren geisoleerd uit menselijke vaten van verschillende oorsprong. Het bleek dat er geen duidelijke verschillen in de groei (deel) snelheid was tussen de verschillende gladdespiercel-types, maar dat er wel duidelijke verschillen in de migratie-snelheid tussen de verschillende gladdespiercellen waren. Ook bleek dat er verschillen in de uitscheiding van u-PA, t-PA en PAI-1 in het cel medium waren. Dit leidde tot verschillen in de mate waarin het plasminogeen activatie systeem een rol speelde in de migratie bij de gladdespiercel-types.

Regulatie van de expressie van plasminogeenactivatoren door cel-dichtheid

Cellen kunnen zich blijven delen totdat het kweekschaaltje helemaal vol zit. De cellen gaan dan langzamer delen en veranderen van vorm. In hoofdstuk 5 laten we zien dat de mate waarin een kweekschaaltje gevuld is met gladdespiercellen van invloed is op de hoeveelheid t-PA en PAI-1 die in het medium van de cellen is terug te vinden. Cellen kunnen t-PA, u-PA en PAI-1 uitscheiden in het medium en ook weer opnemen. Op deze manier wordt de totale proteolytische activiteit door de gladdespiercellen gereguleerd. Dit is ook noodzakelijk want teveel proteolytische activiteit kan ervoor zorgen dat de cellen de hele omliggende matrix afbreken waardoor de cellen hun houvast verliezen. Te weinig proteolytische activiteit zorgt er echter weer voor dat de cellen zich nauwelijks kunnen losmaken en hierdoor niet kunnen migreren. Uit ons onderzoek bleek dat bij een toenemende cel-dichtheid de hoeveelheid t-PA en PAI-1 in het kweekmedium afnam. Dit werd deels veroorzaakt door een toegenomen opname van t-PA en PAI-1 door de cellen via de al eerder genoemde LRP en VLDL-receptoren.

Vergelijking tussen endotheelcellen en gladdespiercellen

Endotheelcellen vormen de binnenbekleding van de vaatwand. In hoofstuk 6 hebben we de migratie en invasie van endotheelcellen en gladdespiercellen met elkaar vergeleken. Er bleken duidelijke verschillen in migratiegedrag te zijn tussen deze twee types vasculaire cellen. Endotheelcellen konden goed door fibrine-lagen migreren en hierbij speelde u-PA en de cel-receptor voor u-PA een belangrijke rol. Echter bij de migratie van endotheelcellen *over* fibrine was het plasminogeen activatie systeem helemaal niet betrokken. Bij gladdespiercellen speelde het plasminogeen activatie-systeem een rol in migratie *over* en *door* een extracellulaire matrix, maar konden gladdespiercellen niet *over* of *door* fibrine heen migreren.

Proteolytische enzymen in de menselijke vaatwand

In hoofdstuk 7 werd een inventarisatie gemaakt van de proteolytische enzymen die voorkomen in de menselijke vaatwand. Zowel het plasminogeen activatie systeem als de matrix metalloproteinases werden bestudeerd met behulp van immunohistochemie. Met deze techniek kan specifiek een eiwit worden aangetoond in hele dunne plakjes vaatwandweefsel. Het bleek dat er verschillen waren in de hoeveelheden proteolytische enzymen en remmers tussen normale en atherosclerotische vaten.

Gen-transfer: remming locale proteolytische activiteit

In hoofdstuk 8 hebben we de mogelijkheid van gentransfer onderzocht om de migratie van gladdespiercellen te beinvloeden. Hiervoor werd een hybride-eiwit ontwikkeld bestaande uit een remmer van plasmine gekoppeld aan het deel van u-PA wat aan de u-PA receptor kan binden. Dit hybride eiwit (ATF.BPTI) kan aan de cel binden via u-PAR en zo dicht bij het cel-oppervlak de proteolytische activiteit van plasmine remmen. Het gen voor dit eiwit werd in een adenovirus gezet. Het bleek dat de migratie door matrix van virus geinfecteerde gladdespiercellen sterker werd geremd dan de migratie van cellen die waren getransfecteerd met een adenovirus dat alleen de remmer van plasmine (BPTI) bevatte. Hieruit blijkt dat juist het sturen van de remmer naar het celoppervlak leidt tot effectieve remming.

Algemene conclusies uit ons onderzoek

Uit ons onderzoek komt naar voren dat proteolytische enzymen (met name het plasminogeen activator systeem) een belangrijke rol spelen in gladdespiercel migratie. Het blijkt verder dat de activiteit van het plasminogeen activatie systeem op een verschillende manieren door de gladdespiercellen wordt gereguleerd. De uiteindelijke activiteit is o.a. afhankelijk van het type gladdespiercel en van de gladdespiercel-dichtheid. Er blijken ook duidelijke verschillen te zijn tussen gladdespiercellen en endotheelcellen. Een mogelijke methode voor het voorkomen van restenose na bypass of dotterbehandeling zou kunnen zijn om de gladdespiercel migratie te remmen via de remming van proteolytische activiteit. Een eerste aanzet hiervoor is gemaakt met het ontwikkelen en testen van een adenoviraal construct dat de locale proteolytische activiteit van plasmine remt.

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Curriculum Vitae

Monique Janine Wijnberg werd geboren op 7 mei 1971 te Alkmaar. In 1989 behaalde zij het diploma Voorbereidend Wetenschappelijk Onderwijs aan het Han Fortmann College in Heerhugowaard. In datzelfde jaar begon zij met de studie Scheikunde aan de Universiteit van Amsterdam. De propedeuse werd behaald in augustus 1990. In het kader van de specialisatie richting Biochemie deed ze een afstudeerstage bij de vakgroep Biochemie van de faculteit Geneeskunde, onder leiding van Dr. S. van Weely. Tevens liep ze een extra stage bij de vakgroep Anatomie en Embryologie, onder leiding van Dr. L. Boon. Het doctoraal examen werd behaald in augustus 1994.

Van augustus 1994 tot augustus 1998 was zij werkzaam als AIO (assistent in opleiding) aan de Universiteit Leiden, gedetacheerd bij het Gaubius Laboratorium, TNO Preventie en Gezondheid in Leiden. Daar werd onder leiding van Dr. J.H. Verheijen en Prof. Dr. P. Brakman het in dit proefschrift beschreven onderzoek met een subsidie van de Nederlandse Hartstichting uitgevoerd. Vanaf januari 1999 was zij werkzaam bij Kendle in Utrecht als trainee. Vanaf augustus 1999 werkt zij via Kendle gedetacheerd bij Searle in Maarssen als CRA (Clinical Research Associate).

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Monique