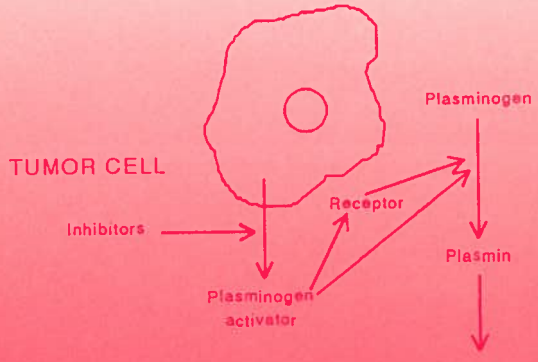
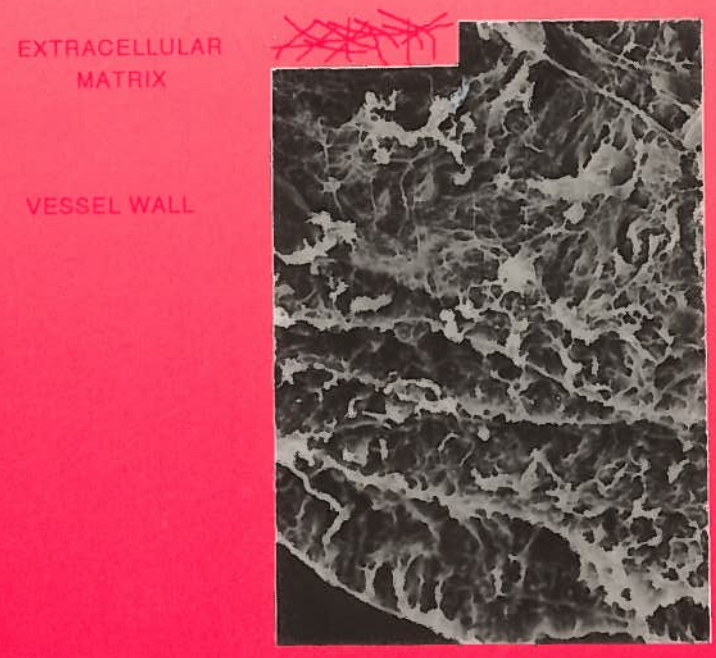
PLASMINOGEN ACTIVATION, EXTRACELLULAR PROTEOLYSIS AND CANCER





PAUL QUAX

PLASMINOGEN ACTIVATION, EXTRACELLULAR PROTEOLYSIS AND CANCER

STELLINGEN

1. De rol van urokinase plasminogeen activator (u-PA) in locale proteolytische processen zoals invasieve groei van tumorcellen en metastase, kan niet los gezien worden van de aanwezigheid van de urokinase receptor.
dit proefschrift.

2. De gladde-spiercellen in de vaatwand, in het bijzonder die van de aorta, moeten beschouwd worden als belangrijke producenten van plasminogeen activator inhibitor type-1 (PAI-1).
dit proefschrift; Sperti et al., Circulation 82, 2274 (1990).

3. De klaring van chylomicron remnants kan in tegenstelling tot VLDL remnants ook geschieden via een andere apolipoprotein-E-specifieke receptor dan de LDL receptor.
Rubinsztein et al., J. Clin. Invest. 86, 1306-1312, (1990); Mulder et al., Biophys. Biochem. Acta (1991) in press.

4. Mignatti et al. hebben gevonden dat een onbekende eiwitfactor, aanwezig in door tumorcellen geconditioneerd medium, in microvasculaire endotheelcellen de synthese induceert van u-PA en basic fibroblast growthfactor, een angiogene factor. De waarneming dat antilichamen tegen deze groeifactor de inductie van u-PA synthese onderdrukken suggereert een rol van u-PA bij de angiogenese van tumoren.
Mignatti et al., submitted for publication.

5. Veel van de teleurstellingen in de biotechnologie kunnen verklaard worden door het feit dat geen rekening is gehouden met de rol van de recent ontdekte chaparone eiwitten bij de eiwitsecretie door bacteriën.
Collier et al., Cell 53, 273-283 (1988); Bieker et al., Trends in Genet. 6, 329-334 (1990); Bieker et al., Cell 61, 833-842 (1990).

6. Het feit dat een enkele puntmutatie in kringle 2 van t-PA de lysine-binding van het t-PA molecuul volledig te niet doet, toont aan dat het bestaan van de door Gething et al. beschreven tweede, onafhankelijke lysine-bindingsplaats in kringle 1 van t-PA hoogst onwaarschijnlijk is.
Weening-Verhoeff et al., Prot. Engineering 4, 191-198 (1990); Gething et al., EMBO J 7, 2731-2740 (1988).

7. Hoewel Owensby et al. suggereren de opname van t-PA door hepatoma cellen te bestuderen, wordt in feite beschreven dat t-PA opname door hepatoma cellen alleen plaatsvindt als t-PA aanwezig is als inactief t-PA:PAI-1 complex. Hiermee tonen de auteurs aan dat dit opnamesysteem niet direct van belang is voor de verwijdering van t-PA activiteit uit het bloed.
Owensby et al., J. Biol. Chem. 264, 18180-18187 (1989).

8. Het gebruik van de term receptor door Stoppelli et al. voor de u-PA bindingplaats aan het celoppervlak blijkt met vooruitziende blik gekozen gezien de ontdekking van de internalisatie van u-PA:PAI complexen.
Stoppelli et al., Proc. Natl. Acad. Sci. 82, 4939-4943 (1985); Cubellis et al., EMBO J. 9, 1079-1085 (1990); Estreicher et al., J. Cell Biol. 111, 783-792 (1990).

9. Een lijst met verklaringen van afkortingen, zoals deze vaak aanwezig is in wetenschappelijke publicaties, zou de leesbaarheid van de advertentiepagina's in dagbladen alsmede van de TNO-krant vergroten.

10. De beste manier om je tanden te laten zien is te glimlachen.

11. Dwarsliggers zijn noodzakelijk om in het rechte spoor te blijven.

Stellingen bij het proefschrift: Plasminogen activation, Extracellular proteolysis and Cancer.

Paul Quax, februari 1991

PLASMINOGEN ACTIVATION, EXTRACELLULAR PROTEOLYSIS AND CANCER

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op donderdag 21 maart 1991
te klokke 16.15 uur

door

PAULUS HUBERTUS ANDREAS QUAX

geboren te Geleen in 1961

"ALL IN" BV - KATWIJK

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The study presented in this thesis was performed at the Gaubius Institute TNO, Leiden (head: Prof.Dr. P. Brakman), and supported by a "TNO Beleidsruimtesubsidie".
Financial support by the Gaubius Institute TNO, Leiden, KABI Diagnostica, endotoxin unit, and Pharmacia LKB Biotechnology for the publication of this thesis is gratefully acknowledged.

veur Pap en Mam
aan Rafaëlle

CONTENTS

CONTENTS (continued)

CHAPTER 1

GENERAL INTRODUCTION

In many biological processes migration of cells and remodeling of tissue occurs. These may be either physiological processes, like neuron growth, ovulation and embryogenesis, or pathological processes, like tumor cell invasion and metastasis. During these events the cells may facilitate the migration within a tissue or through the extracellular matrix and basement membrane by localized proteolysis.

The involvement of limited extracellular proteolysis in cell migration has been well established (Danø, 1985; Tryggvason, 1987; Mignatti, 1986; Rifkin, 1989). A large number of proteolytic enzymes may be involved in these processes. The metalloproteinases stromelysin and collagenase (Trygvasson, 1987; Murphy, 1989;), esp. collagenase type IV (Liotta, 1986; Reich, 1988), cathepsins (Weiss, 1990; Montcourrier, 1990) elastase (Machovich, 1989) and plasmin are proteolytic enzymes found to be involved in cell migration and in degradation of extracellular matrix. It is suggested that these enzymes function as a kind of proteolytic cascade. Plasmin, or more specifically the activation of its zymogen plasminogen by specific plasminogen activators, is thought to have a triggering function in this proteolytic cascade since plasmin may not only degrade components of the extracellular matrix directly but it can also activate pro-enzymes of other proteases (Murphy, 1989; Rifkin, 1989). Inhibition of the activation of plasminogen has been shown to block cell migration effectively (Ossowski, 1983; Mignatti, 1986). Regulation of the plasminogen activator activity may therefore be a key process in the regulation of the extracellular proteolysis.

In this introduction an outline of the various components of the plasminogen activation system, plasmin and its zymogen plasminogen, plasminogen activators, plasminogen activator inhibitors and receptors, will be given. Furthermore, since the plasminogen activation system is thought to be involved in a broad spectrum of processes, both physiological and pathological, in which limited extracellular proteolysis occurs, a survey will be given on the involvement of the plasminogen activation system in this kind of processes.

Plasminogen/Plasmin.

Plasminogen is a single chain circulating glycoprotein of 90 kD, its normal plasma concentration being 2.2 μM (Mayer, 1990). Plasminogen is also found to be present outside the blood circulation within the tissue. Plasminogen can be converted to the active serine protease plasmin by specific plasminogen activators that cleave the Arg-560:Val-561 bond in the single chain plasminogen (Robbins, 1967). The plasmin thus formed consists of a light chain and a heavy chain linked by disulphide bonds. The light chain contains the proteolytic domain which is similar to that of other serine proteases. The heavy chain contains five homologous kringle structures, of approximately 10-12 kD each, and is thought to mediate the binding to lysine, fibrinogen, fibrin and related compounds (Castellino, 1988).

Plasmin is a trypsin-like serine protease with a broad specificity. Besides fibrin it degrades many other proteins including extracellular matrix components. Plasminogen can bind to the extracellular matrix in a specific, saturable and reversible manner. This matrix bound plasminogen can still be converted to plasmin, which remains associated with the matrix (Knudsen, 1986; Mayer, 1990). Plasminogen can also bind to a specific receptor on the cell surface (Plow, 1986; Stephens, 1989).

Plasminogen activators.

The conversion of the zymogen plasminogen to active plasmin is catalyzed by specific enzymes, known as plasminogen activators. Two distinct plasminogen activators are known, tissue-type (t-PA) and urokinase-type (u-PA). These two enzymes are different but related in structure and function. They are encoded by different genes located on chromosome 8 (Verheijen, 1986) and 10 (Triputti, 1985) for t-PA and u-PA respectively. Both enzymes are synthesized as single chain proteins. Single chain u-PA is a pro-enzyme whereas single chain t-PA is an active enzyme. The single chain enzymes can be cleaved by plasmin and the resulting two chains remain connected by disulphide bonds. Both enzymes contain a protease domain similar to that of other serine proteases. Also in the non-catalytic chain similarities are found, they both contain a growth factor domain, homologous to epidermal growth factor, and kringle structures like in plasminogen. u-PA has only one kringle whereas t-PA has an additional kringle and at the aminoterminal part of the protein a finger domain similar to structures found in fibronectin. Apart from structural differences also differences in enzymatic characteristics exist. An important difference is that t-PA is fibrin specific. It binds to fibrin and its activity is stimulated by this binding (Verheijen ,1982; Hoylaerts, 1982), whereas u-PA does not bind to fibrin nor is its activity stimulated by fibrin. Therefore it was thought that the function of t-PA is mainly in fibrinolysis, whereas u-PA seemed to be important in other extracellular proteolytic processes such as those involved in cell migration, tissue remodeling and more specifically tumor development (Danø, 1988). Recently this sharp distinction has

been abandoned, since many tumor cell lines were found to produce besides u-PA also t-PA (Cajot, 1986) suggesting a possible role for t-PA in tumor development.

Plasminogen activator inhibitors.

The activity of plasminogen activators can be inhibited by specific inhibitors. Three types of inhibitors have been described, plasminogen activator inhibitor type-1 (PAI-1), type-2 (PAI-2) and protease nexin (Sprengers, 1987; Kruithof, 1988a). PAI-1, a 50 kD glycoprotein, forms inactive complexes with single chain and two chain t-PA as well as two chain u-PA but not with pro-u-PA. PAI-2, a 46 kD serine protease inhibitor, forms inactive complexes with both t-PA, single chain as well as two chain, and u-PA, but reacts more readily with u-PA. The reactivity of PAI-2 with two chain t-PA is higher than with single chain t-PA (Kruithof, 1988b).

The genes for PAI-1 and PAI-2 are located on chromosome 7 (Klinger, 1987) and 18 (Antalis, 1988) respectively. A third type of inhibitor is Protease Nexin, a 51 kD glycoprotein that inhibits a variety of serine proteases (Scott, 1985). Its reactivity with plasminogen activators is less than that of PAI-1 and PAI-2.

Urokinase receptor.

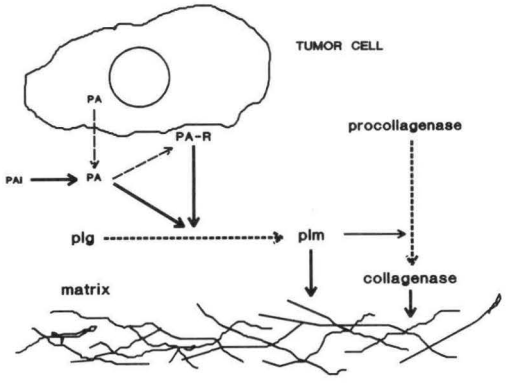
Recently binding of u-PA to the cell surface has been demonstrated (Vassalli, 1985; Stopelli, 1985). u-PA can bind to a specific cell surface binding site without affecting the proteolytic activity (Cubellis, 1986). The receptor binding site is located at the growth factor domain of u-PA. Pro-u-PA can bind to the receptor as well as active two chain u-PA can. When bound to the receptor pro-u-PA can still be activated (for review see: Blasi, 1988). The cDNA of the u-PA receptor has recently been cloned (Roldan, 1990), and the u-PA receptor has been characterized as a 55 kD protein (Nielsen, 1988; Behrendt, 1990) attached to the cell membrane via a glycolipid anchor (Ploug, 1991). Only very recently it has been reported that both PAI-1 (Cubellis, 1990) and PAI-2 (Estreicher, 1990) can interact with receptor bound u-PA. The formed complexes are internalized. After the u-PA:PAI complexes are internalized, u-PA and the inhibitor are degraded (Cubellis, 1990; Estreicher, 1990). Whether the receptor itself is also degraded in the internalization process remains to be determined. Receptor binding of u-PA or activation of receptor bound pro-u-PA (Ellis, 1989) localizes the plasminogen activation at the cell surface, which may be an advantage for cells during extracellular proteolytic processes.

Plasminogen activation and extracellular proteolysis.

Plasmin is a broad spectrum serine protease. Because of its relatively low substrate specificity plasmin is thought to be involved in many proteolytic processes. The role of the plasminogen activation system in fibrinolysis is studied most extensively. Because of

its binding to both plasminogen and fibrin and the stimulation of its activity upon binding (Verheijen, 1982; Hoylaerts, 1982), t-PA is thought to be the major plasminogen activator responsible for fibrinolysis. Recently it has been found that u-PA is ubiquitous in blood in concentrations comparable to t-PA (Kluft, 1984). This might suggest an involvement of both plasminogen activators in fibrinolysis (for a review see: Kluft, 1988).

In many processes where remodeling of tissues occurs, it is a result of migration of cells, like in embryogenesis and in the ovulation process. Cell migration involves, as indicated above, a cascade of proteolytic events. Tumor cells, for instance, have to migrate through the vessel wall when they enter the bloodcirculation in order to form distant metastasis. The vessel wall, including the extracellular matrix and the basement membrane, is degraded proteolytically during this process.
How the various components of the plasminogen activation system may be involved in the cell migration process is indicated in the simplified model in figure 1.



The migrating cell produces plasminogen activator(s), which may be present either in its single chain or two chain form. When secreted, it can bind to its specific cell surface receptor. For u-PA the single chain pro-enzyme can be activated when receptor bound. The active plasminogen activator converts the zymogen plasminogen into active plasmin. Plasmin then degrades components of the extracellular matrix either directly or indirectly by the activation of pro-enzymes of other proteases like pro-collagenase. The activity of the plasminogen activator can be regulated by the specific inhibitors. Plasminogen activators as well as plasminogen might be bound to their specific receptors, and then the process of plasminogen activation will occur at the cell surface, leading to a very localized proteolytic activity. The localization of the proteolytic activity at the close contact sites between cells and extracellular matrix substratum may be advantageous for

the migrating cells, since the inhibitory effect of e.g. α2-antiplasmin or even PAI can be circumvented.

At the beginning of this century the first experiments on cell and tissue culturing were performed. In this early period of culturing techniques cells and tissues were frequently cultured on blood and plasma clots. It was observed that culturing of some tissues led to lysis of the clot, while other tissues had no effect on the clot consistency (Fleisher, 1915). Fisher demonstrated that dead muscular tissue in a Rous sarcoma culture was invaded by sarcoma cells, while the plasma clot on which the culture was grown, was lysed (Fisher, 1925). The role of fibrinolysis in tissue repair can, in retrospect, be seen in the observations of Loeb who found that epithelial cells migrate through the scab covering an injury while liquifying the coagulum (Loeb, 1898). Although the role of fibrinolysis in cell migration and tissue remodeling was suggested already at the beginning, it is only recently that this important feature has gained the interest of many scientist.

The number of processes described in which plasminogen activator mediated extracellular proteolysis plays a role is rapidly increasing. A few of these processes will be described in more detail.

The involvement of the plasminogen activation system in ovulation has been reported (Canipari, 1987; Liu, 1987; Hsueh, 1988; Cajander, 1989; Tsafriri, 1989). Just prior to ovulation and rupture of the follicle wall, elevated levels of plasminogen activators were detected, leading to plasmin formation and degradation of the follicle wall.

Elevated levels of plasminogen activators have been observed also at the leading edge of an extending neuron (van Evencooren, 1987; Erickson, 1989; Menoud, 1989) indicating the involvement of plasminogen activators in neuron growth.

Angiogenesis, the formation of new blood vessels, involves endothelial cell migration. The role of the plasminogen activator mediated proteolytic processes in angiogenesis has been demonstrated by several authors (Goldfarb, 1986; Moscatelli, 1988; Montesano, 1990; Pepper, 1990). Also in keratinocyte migration during wound healing (Grøndahl-Hansen, 1988; Wilkinson, 1989; Delrosso, 1990) and trophoblast implantation during embryogenesis (Sappino, 1989, Feinberg, 1989) the involvement of the plasminogen activator system in extracellular proteolysis during cell migration was clearly demonstrated.

It has been suggested that smooth muscle cell migration after vessel wall injury is mediated by plasminogen activation, since the u-PA levels in the vessel wall were markedly increased after vessel wall injury by de-endothelization (Clowes, 1990).

The role of proteolytic enzymes in muscle cell differentiation and regeneration processes has recently been suggested. Plasminogen activator levels have been reported to be

increased in muscle cell differentiation (Barlovatz-Meimon, 1990; Festhoff, 1990; Hantai, 1990).

Not only in this kind of, more or less, physiological processes but also in pathological processes, like metastasis, plasminogen activation may be involved. The role of the plasminogen activation system in metastasis and tumor growth has been studied extensively (for reviews see: Danø, 1985; Markus, 1988; Danø, 1988). A role in regulating tumor growth and invasiveness has been proposed for plasminogen activators on the basis of the generally observed increase in plasminogen activator concentrations in several tumors (Danø, 1985, 1988) and tumor cell lines in culture when compared with normal cells (Hoal-van Helden, 1986; Cajot, 1986; Stephens, 1987; Sitrin, 1990). However, direct evidence for the involvement of plasminogen activators in invasive growth of tumor cells is scarce. In a limited number of studies antibodies against u-PA were shown to inhibit invasive growth (Ossowski, 1983, 1988; Mignatti, 1986) pointing to a direct role of the plasminogen activators in tumor cell invasion. Degradation of the extracellular matrix by tumor cells is an essential step in metastasis. Plasminogen activation is one of the first steps in the cascade of proteolytic reactions occurring in extracellular matrix degradation (Rifkin, 1989). The regulation of the plasminogen activator activity might thus be a very crucial step in metastasis and invasive growth.

Aims and outlines of the studies.

Plasminogen activator activity can be regulated in several ways, at the level of protein production or the enzymatic activity. The protein production might be regulated at the mRNA level by mRNA synthesis and stability, or at the translation and protein synthesis level. Regulation of the activity of the enzymes may occur at many levels: at the level of activation of proenzymes, at the level of interaction of plasminogen activators with their inhibitors, activation or inactivation of inhibitors, and at the level of secretion of the plasminogen activators, localization by cell surface binding to specific receptors, secretion and internalization.

In order to study the regulation of plasminogen activation in vivo the levels of plasminogen activator activity as well as the levels for t-PA, u-PA and PAI-1 mRNA in various tissues of control and endotoxin treated rats were determined. It was reported by Emeis and Kooistra that endotoxin had a strong effect the plasma PAI activity in rats (Emeis, 1986). Moreover, an increase in PAI-1 mRNA was observed in cultured endothelial cells after endotoxin treatment (van den Berg, 1988). The modulation of the plasminogen activator activity by endotoxin can be used to study the regulation of the plasminogen activator activity in vivo at the mRNA level. This analysis was performed

in various rat tissues and in the different cell types of the liver as described in Chapter 2.

Of special interest was the effect of endotoxin on the plasminogen activator activity in the vessel wall. In Chapter 3 the regulation of the plasminogen activator activity in the different cell types of the rat aorta is studied in detail. From endotoxin treated rats and control rats the plasminogen activator activity as well as the inhibitor activity and the mRNA levels were analyzed in the various layers of the aorta, the adventitia, the media and the intima, each containing distinct cell types.

The production and the regulation of production of plasminogen activators and their inhibitors in tumor cells is studied in Chapter 4. For twenty-two human tumor cell lines of different origin the t-PA, u-PA, PAI-1 and PAI-2 antigen and mRNA levels were measured and the possible levels on which regulation can take place were determined.

The correlation between production of specific components of the plasminogen activator system and proteolytic degradation of extracellular matrix in vitro with in vivo metastasis formation in nude mice was studied for a set of human melanoma cell lines and is described in Chapter 5.

Chapter 6 reports a study on the causal involvement of the plasminogen activator system in proteolytic extracellular matrix degradation. Non-proteolytic mouse L-cells were transfected with the genes of human u-PA or u-PA receptor and the effect of the subsequently produced components, as well as their interaction, on extracellular matrix degradation in vitro was determined.

The involvement of the plasminogen activator system in a physiological extracellular proteolytic process, the differentiation of muscle cells including the formation of myotubes, is studied in Chapter 7. The regulation of the components of the plasminogen activator system during in vitro muscle cell differentiation was analyzed and a model for the interactions of the various components was proposed.

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CHAPTER 2

ENDOTOXIN INDUCTION OF PLASMINOGEN ACTIVATOR AND PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 mRNA IN RAT TISSUES IN VIVO

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Journal of Biological Chemistry (1990) 265; 15560-15563

ABSTRACT

The tissue specific distribution of tissue-type and urokinase-type plasminogen activator (t-PA and u-PA) and their inhibitor type 1 (PAI-1) was analyzed at mRNA level in five major rat organ tissues. t-PA mRNA was detected in lung, kidney, heart and liver. u-PA mRNA was detected in kidney and lung. Presence of PA mRNA correlated with the detection of PA activity in extracts of these tissues. PAI-1 mRNA was detected predominantly in heart and lung. Although PAI activity could not be measured directly in tissue extracts, the presence of PAI-1 mRNA correlated with the occurrence of PA-PAI complex in fibrin autography of tissue extracts.

Endotoxin injection caused a very large increase in plasma PAI activity. This increase correlated with a marked increase in PAI-1 mRNA in nearly all tissues studied. The increase in PAI-1 mRNA is most pronounced in lung and liver. Endotoxin injection also caused an increased level of t-PA mRNA in heart and kidney, and an increased u-PA mRNA level in kidney. mRNA analysis of freshly isolated and separated subfractionated liver cells showed that the marked increase in PAI-1 mRNA in the liver after endotoxin injection may be due mainly to a strong increase of PAI-1 mRNA in the liver endothelial cells.

INTRODUCTION

Plasminogen activators are serine proteases that can activate the zymogen plasminogen to the active serine protease plasmin. The protease plasmin has a broad spectrum of activities and is thought to be involved in many processes where extracellular proteolysis occurs. Its role in fibrinolysis has been studied most extensively but plasmin may also play a role in processes such as cell migration, tissue remodelling, tumor development and metastasis, and angiogenesis (1).

Two types of plasminogen activators can be distinguished, tissue-type plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA), which differ in immunological reactivity, and are encoded by different genes (2-4).

The activity of plasminogen activators can be inhibited by specific inhibitors. At least two types of inhibitor exist, plasminogen activator inhibitor type 1 (PAI-1) and type 2 (PAI-2), encoded by different genes (5). Both PAI-1 and PAI-2 can form proteolytically inactive stable complexes with t-PA or u-PA.

It has previously been reported that treatment of rats with endotoxin or interleukin 1, increased plasma PAI activity rapidly (6,7). A similar effect has been observed in cultured endothelial cells treated with endotoxin. In this case an increase in PAI-1 mRNA was observed (8).

In contrast to the situation in cultured endothelial cells, no effect on the PAI-1 level has been found in endotoxin treated cultured hepatocytes (6,8). PAI-1 production by hepatocytes can be induced by other mediators such as glucocorticoids or cyclic AMP, however (9-11). Although the tissue distribution of plasminogen activator activity has been investigated (12,13) as well as the t-PA mRNA levels in several murine tissues (14), the combination of the mRNA levels and activity of PA has to our knowledge not yet been demonstrated.

In this paper we have analyzed the mRNA levels of t-PA, u-PA and PAI-1 and the plasminogen activator activities in several tissues of the rat. In addition the effect of endotoxin treatment of the rat on these mRNA and activity levels was studied. The effect of endotoxin treatment on the PAI-1 mRNA level in the liver was studied in more detail. The PAI-1 mRNA levels were determined in endothelial cells, Kupffer cells and parenchymal cells (hepatocytes) isolated from the liver of endotoxin treated rats.

MATERIALS AND METHODS

Animal experiments

For all animal experiments male Wistar rats (280-320 gr), obtained from the Broekman Institute, Helmond, The Netherlands, were used. All experiments were performed under Nembutal (Sanofi, France) anaesthesia (60 mg/kg; intraperitoneally).

Endotoxin treatment

Rats were intravenously injected with endotoxin (E.coli lipopolysaccharide LPS, serotype 0128:B12, prepared by phenol extraction, Sigma, USA) 50 μg/kg in a volume of 1 ml/kg. At the times specified, blood was obtained by aortic puncture, and anti-coagulated with 0.1 volume of trisodium citrate (0.13 M). Platelet-free plasma was prepared at 4 °C and stored at -20 °C.

Tissues were obtained immediately after bleeding, briefly washed in saline and snap frozen in liquid nitrogen. Tissues were stored in air-tight containers at -70 °C.

Plasma PAI activity

Plasma PAI activity was determined by titration of (diluted) citrated plasma with human 2-chain melanoma t-PA, followed by the spectrophotometric determination of residual t-PA activity, as described (15).

Tissue PA activities

Frozen tissues, pooled from two rats, were weighed, triturated in liquid nitrogen and resuspended (at 40 mg/ml) in extraction buffer (16) for all tissues except kidney, which was resuspended in 0.5 M KSCN. The tissue suspensions were then homogenized at 4 °C,

using a polytron PTA7 homogenizer for 1 min at full speed, and the extracts centrifuged. PA activities in the supernatant were then determined spectrophotometrically essentially as described (17), using human t-PA as a standard. The plasminogen activator activity was determined in the presence and absence of anti-rat t-PA IgG. The difference between these activities was regarded as t-PA activity. Fibrin autography of tissue extracts was performed as described by Granelli-Piperno and Reich (18).

Liver cell isolation

Parenchymal liver cells were isolated from rats after a short collagenase (type IV, 0.05%, Sigma, USA) perfusion (5 min) of the liver by the method of Seglen (19), modified as described by Kuiper et al. (20).

Endothelial and Kupffer cells were isolated after collagenase (type I, 0.05%, Sigma, USA) perfusion of the liver at 37°C and subsequent counterflow centrifugation, essentially as described by Nagelkerke (21) except for a replacement of the first elutriation step by a centrifugation step of 2 min, 75 g (20). The purity of the cell fractions was routinely checked using light-microscopy. 3,3'-diaminobenzidine peroxidase staining followed by Papanicolaou counterstaining was used to discriminate between Kupffer cells and endothelial cells (21). Staining with 1,1'-dioctadecyl 3,3,3',3'-tetramethylindocarbocyanine perchlorate acetyl low density lipoprotein was used to identify the endothelial cells (22). Using these methods it was found that the parenchymal cell fraction was pure for more than 99.5%, the Kupffer cell fraction was pure for more than 90% and in the endothelial cell fraction no other cells could be detected.

The validity of these staining methods for determination of the purity of the isolated cell fractions has been demonstrated previously by the identification of the pattern of eicosanoid production in the liver cell fractions (23).

RNA isolation

Tissues or cells were homogenized in 4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.5, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol. RNA was isolated from the homogenate according to Chomczynski and Sacchi (24). Total RNA was quantified by absorbance at 260 nm. Poly A^+ RNA was prepared by affinity chromatography, using oligo-dT cellulose (type 77F, Pharmacia, Sweden).

mRNA analysis

The following cDNA fragments were used as probes in the hybridization experiments: a 1.9 kb BglII fragment of the human t-PA cDNA (25), a 1.0 kb EcoRI PstI fragment of the human u-PA cDNA (26), a 0.9 kb PvuII fragment of the rat PAI-1 cDNA (27), a 1.2 kb EcoRI fragment of the human PAI-2 cDNA (kindly provided by Dr. E.K.O. Kruithof)

(28) and a 1.2 kb PstI fragment of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cDNA (kindly provided by Dr. R. Offringa, 29). The cDNA fragments were labelled with ^{32}P dCTP (Amersham, UK) using the random primer method (Multiprime, Amersham, UK).

RNA samples were electrophoresed on a 1.2% agarose gel containing 7.5% formaldehyde. The RNA's were transferred to a nylon membrane (Hybond N, Amersham, UK) using a Vacugene system (Pharmacia, Sweden).

The membranes were hybridized at 60°C in a solution containing 7% SDS, 0.5 M $NaHPO_4$ pH 7.2, 10 mM EDTA with labeled cDNA fragments. The blots were routinely washed with 2xSSC, 1% SDS for 1 h at 60°C (1xSSC = 0.15 M NaCl, 0.015 M sodium citrate). Autoradiograms were prepared using Kodak XAR 5 film and intensifying screens at -70°C.

RESULTS

Effect of endotoxin on plasma PA inhibitor activity

As reported previously (6), the injection of rats with endotoxin induced a rapid and dosage dependent increase in plasma PAI activity. Here, the plasma PAI activity was analyzed at an endotoxin dosage of 50 μg/kg, in order to determine the optimal time after endotoxin injection for mRNA analysis. Peak plasma levels (about 40-fold increased above base line levels) were seen at 4 hrs (Fig. 1). As the most rapid increase in PA inhibitor activity occurred between 2 and 4 hours, it is likely that mRNA levels reached their maximum during this time period. For this reason tissue samples for mRNA analysis were collected at 3 hrs after endotoxin injection.

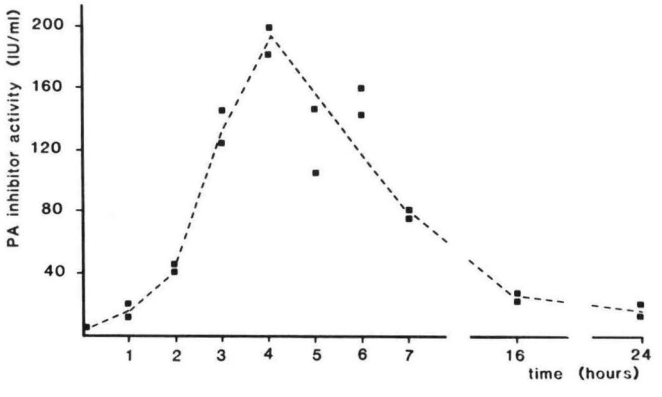

Figure 1. PAI activity in plasma of endotoxin treated rats.
At t = 0 endotoxin was injected at a dose of 50 μg/kg. During 24 h after endotoxin injection PAI activity was determined as described in Materials and Methods and expressed in IU per ml.

mRNA level in organs before and after endotoxin treatment

The mRNA levels for t-PA, u-PA and PAI-1 were determined in lung, liver, kidney, heart and skeletal muscle. RNA from these tissues was subjected to electrophoresis on denaturing formaldehyde agarose gels, transferred to nylon membranes and hybridized with ^{32}P dCTP labelled cDNA probes. In Fig. 2 the results are shown. No significant degradation of RNA had occurred and good hybridization signals could be detected, although for t-PA and u-PA heterologous probes were used. No cross-hybridization occurred between the various probes.

Under basal conditions (lanes C in Fig. 2), t-PA mRNA was detected in lung, liver, kidney and heart tissue. The strongest hybridization signal was found in lung tissue. Intermediate signals were detected in liver and kidney tissue. In heart tissue only a very weak signal could be detected. For u-PA mRNA a strong signal was detected in kidney and a very weak signal in lung. PAI-1 mRNA could be detected in lung and heart tissue (Fig. 2). PAI-2 mRNA could not be detected in the tissues studied using a human cDNA fragment as a probe. The possible origin of the observed increase of plasma PAI activity after endotoxin injection was studied by analysis of the mRNA levels of PAI-1 in the various tissues. In Fig. 2, lanes E, it is shown that 3 hrs after endotoxin injection the PAI-1 mRNA level increased in all the tissues studied. The increase was most pronounced

Table 1. t-PA activity in tissue extracts (IU/g wet weight).

	Controls	Endotoxin treated	Change in activity
Lung	1826	2165	+19%
Liver	28	3	-89%
Kidney	231	93	-60%
Heart	238	115	-52%
Muscle	33	20	-39%
Aorta	95	28	-71%

Tissue-type plasminogen activator (t-PA) activity in tissue extracts of control rats and endotoxin treated rats. Endotoxin (50 μg/kg) was injected 3 h before killing of the rat and isolation of the tissues. t-PA activity was determined spectrophotometrically. Human two-chain melanoma t-PA was used as a standard. t-PA activity was determined by the difference in PA activity in the presence and the absence of anti-rat t-PA antibodies.

in lung and liver tissue. These results indicated that the increase of plasma PAI activity is paralleled by a detectable increase in PAI-1 mRNA levels. PAI-2 mRNA was not detected after endotoxin treatment in the studied tissues.

When analyzed at the same time interval after endotoxin injection, surprisingly, t-PA mRNA levels also appeared to be increased in kidney and heart tissue, while the u-PA

mRNA in kidney tissue was also slightly increased, although the control GAPDH mRNA showed little variations.

Plasminogen activator activity in tissue extracts before and after endotoxin treatment

Plasminogen activator activity was determined in extracts from six tissues (lung, liver, kidney, heart, skeletal muscle and aorta), obtained from control and endotoxin treated (50 μg/kg, 3 hrs) rats (Table 1). We were not able to isolate sufficient quantities of RNA from the aorta for mRNA analysis. In all other tissues, except kidney, the predominant plasminogen activator was t-PA, as demonstrated by antibody-quenching experiments (85-97%)(data not shown) and fibrin autography (Fig.3).

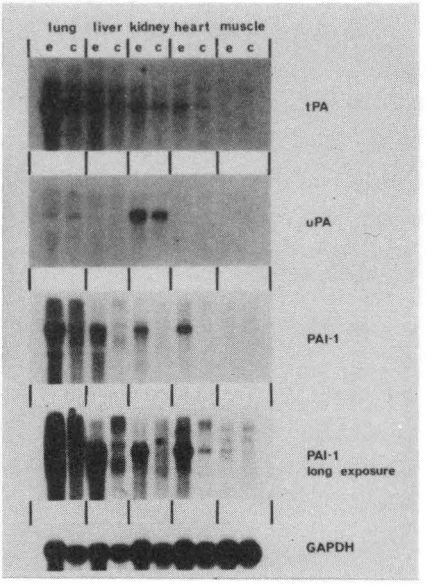

Figure 2. Northern blots of RNA extracted from different tissues of control (C) rats and rats treated with endotoxin (E).
Endotoxin (50 μg/kg) was injected 3 hours before killing and RNA extraction. Poly A^+ RNA isolated from 50 μg total RNA was used in every lane. The filters were subsequently hybridized with DNA fragments of human t-PA, human u-PA, a rat PAI-1, and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

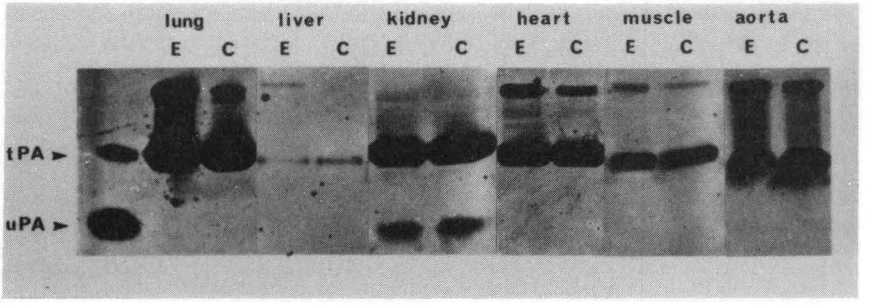

Figure 3. Fibrin autography of extracts from different tissues of control (C) rats and endotoxin (E) treated rats. Endotoxin (50 μg/kg) was injected 3 hours before killing and tissue extraction. Equal quantities of sample for each tissue before and after endotoxin were used but sample volumes of different tissues were adjusted to give lysis zones of comparable intensities. Rat urine, containing t-PA and u-PA was used as a standard.

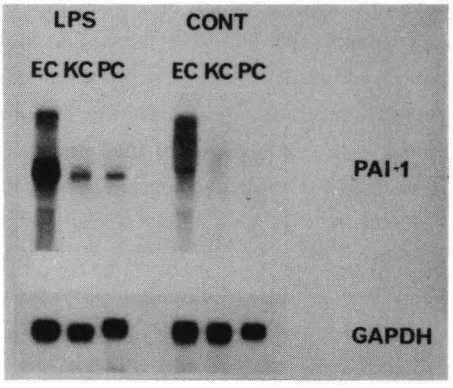

Figure 4. PAI-1 mRNA in various cell types of rat liver.
Three hours after endotoxin (LPS) injection in rats, the livers were perfused and various cell types were separated: endothelial cells (EC), Kuppfer cells (KC) and parenchymal cells (PC). RNA was isolated and hybridized with rat PAI-1 cDNA. The lower panel shows the same blot hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

After endotoxin treatment, in lung a small increase in t-PA activity was observed, while in all other tissues the t-PA activity was markedly decreased. Non-t-PA activity, as determined using anti t-PA antibodies in the assay, remained fairly constant (data not shown).

PAI-1 mRNA in different liver cell types before and after endotoxin treatment

In the liver a strong increase in PAI-1 mRNA was observed after endotoxin injection (Fig. 2). As no influence of endotoxin on PAI-1 expression on cultured hepatocytes was found (6), we investigated which cell type in the liver is responsible in vivo for the endotoxin effect on PAI-1 expression. The presence of PAI-1 mRNA was determined in the various liver cell types of control rats and of rats after endotoxin injection (Fig. 4). Under normal conditions PAI-1 mRNA could be detected in liver endothelial cells only. After endotoxin injection a very strong increase of the PAI-1 mRNA level was seen in the endothelial cell fraction and, moreover, PAI-1 mRNA could be detected in the parenchymal (hepatocytes) cell fraction and in the Kupffer cell fraction. These results suggest that mainly the endothelial cells are responsible for the effect of endotoxin injection on the increase of PAI-1 mRNA in the liver. t-PA mRNA was below the detection limit in these RNA samples.

DISCUSSION

The specific mRNAs of the plasminogen activators, t-PA and u-PA, and their inhibitors, PAI-1 and PAI-2, were determined in major rat organ tissues. t-PA mRNA was found in the lung, the liver and kidney and the heart. u-PA mRNA could only be detected in kidney and lung tissue. In muscle neither t-PA nor u-PA could be detected. PAI-1 mRNA could, under basal conditions, be detected in lung and heart tissue. PAI-2 mRNA could not be detected in the tissues studied. It can not be excluded that this is due to a lack of homology between rat PAI-2 mRNA and the human cDNA probe used. The difference in t-PA and PAI-1 mRNA occurrence in rat and human liver is striking. In rat liver t-PA mRNA was detected but no PAI-1 mRNA while in human liver PAI-1 mRNA but no t-PA mRNA was detected (9,30). Analysis of the PAI-1 mRNA in the rat showed only one transcript for PAI-1, in contrast to human and bovine PAI-1 (5,31). This is in accordance with the PAI-1 RNA analysis in cultured rat HTC cells (10).

Rat tissues were also analyzed for the presence of PA-activity. The tissue specific distribution of PA and PAI mRNA is consistent with the presence of PA-activity or PA-PAI complexes (as detected using fibrin autography). The presence of the mRNA may give a good indication for the occurrence of the corresponding protein. But as can be seen from our data, Table 1 and Fig. 2, in tissues with comparable mRNA levels for t-PA (liver and kidney), very different t-PA activities were observed. This indicates that besides regulation of mRNA levels other regulatory mechanisms are involved, such as pro-enzyme activation (2), receptor binding (32,33), complex formation with inhibitors (5) or translational control of the production of t-PA, u-PA, PAI-1 and PAI-2 (34).

At 3 hrs after endotoxin injection the plasma PAI activity was steeply increasing and had not yet reached its maximum. PAI-1 mRNA analyzed at the same time point was markedly increased in nearly all the tissues studied. This increase was most pronounced in lung and liver tissue. Thus, the increase in plasma PAI activity after endotoxin injection is most likely caused by increase of PAI-1 mRNA.

Unexpectedly, the mRNA levels for t-PA (in kidney and heart) and to a lesser extent u-PA (in kidney) also increased after endotoxin injection. The increase in PAI-1 mRNA levels is in agreement with the detection of more PA-PAI complexes in the fibrin autography experiments. These observations, increase of PAI-1 mRNA and increase of t-PA and u-PA mRNA after endotoxin injection in vivo have, to our knowledge, not been reported before, although recently a rapid increase in plasma t-PA antigen level after endotoxin injection was described (35).

It is striking that the PAI-1 mRNA increase is most pronounced in those organs which are known to be extensively vascularized, such as the lung and the liver. This might suggest that endothelial cells are involved in the PAI-1 increase after endotoxin injection.

This possibility was studied in more detail by subfractionation of the various liver cell types and analysis of the increase in PAI-1 mRNA levels in these liver cell types. Subfractionation of the liver in parenchymal cells (hepatocytes), endothelial cells and Kupffer cells provides a powerful tool to locate the cellular sites of mRNA synthesis in vivo. After the rat was injected with endotoxin, the liver cells remain in their natural environment, and are able to exert their cell to cell contacts, until they are harvested for RNA extraction.

A marked increase in PAI-1 mRNA was seen in the liver endothelial cells but also in the Kupffer and parenchymal cells some PAI-1 mRNA could be detected after endotoxin injection. The detection of PAI-1 mRNA in the Kupffer cell fraction is probably due to a slight contamination (< 10%) with endothelial cells. The parenchymal cell fraction was nearly 100% pure, therefore the detected PAI-1 mRNA is likely to be produced in these cells (20-23). The experiments showed that in vivo, at least in the liver, the endothelial cells are mainly responsible for the strong increase in PAI-1 after endotoxin injection (Fig. 4), but a minor contribution of the hepatocytes can not be excluded. In contrast to the in vivo situation, both cultured liver endothelial cells and hepatocytes produce PAI-1 (9,36). It cannot be concluded from our data that the results obtained with liver endothelial cells can be extrapolated to vascular endothelial cells, although the data might suggest this. The rapid increase in plasma PAI activity after endotoxin injection suggests an acute phase response. A classical acute phase response is thought to be restricted to hepatocytes (37). The increase in PAI-1 mRNA is seen in many tissues, and not limited to the liver. Furthermore, in the liver the increase is mainly due to endothelial cells and not hepatocytes. Therefore the increase of PAI-1 after endotoxin injection cannot be regarded as a classical acute phase response.

In this study we have demonstrated that the tissue-specific distribution of t-PA, u-PA and PAI-1 mRNA correlates with the presence of PA activity and PA-PAI complexes in these tissues. We also showed that the sharp increase in plasma PAI activity after endotoxin injection is due to a sharp increase in PAI-1 mRNA, while t-PA and u-PA mRNA levels are increased as well. The increase in PAI-1 mRNA in the liver in vivo can be mainly ascribed to an increase of PAI-1 mRNA in the endothelial cells.

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CHAPTER 3

PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 IN NORMAL AND ENDOTOXIN TREATED RAT AORTA

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Submitted for publication.

ABSTRACT

The plasminogen activator (PA) and plasminogen activator inhibitor (PAI) in the different layers of the rat aorta were analyzed at the protein and mRNA level. The effect of endotoxin on these components was studied. The PA activity in aorta was identified as tissue-type PA (t-PA) activity. t-PA activity was detected in the adventitia, while PAI activity was detected in the smooth muscle cell containing media. Removal of the endothelium had no or very little effect on PAI activity. t-PA antigen and mRNA were found in both adventitia and media and was not affected by endotoxin treatment. PAI-1 mRNA was found in the media, with or without endothelium. After endotoxin treatment PAI-1 mRNA was strongly increased in the media as well as the adventitia.
It is concluded that the decrease in PA activity in the aorta after endotoxin treatment of rats in vivo is due to an increase in PAI-1 (activity and mRNA), while t-PA antigen and mRNA levels are not affected. The aortic smooth muscle cells produce considerable amounts of PAI-1 (mRNA) in vivo. After endotoxin treatment also adventitia cells, most likely fibroblasts, produce PAI-1 mRNA.

INTRODUCTION

Plasminogen is a zymogen that can be converted to the active serine protease plasmin by plasminogen activators. Plasmin is involved in a broad spectrum of extracellular proteolytic processes including fibrinolysis, tumor cell invasion, and cell migration (1). Two types of plasminogen activators are described, tissue-type (t-PA) and urokinase-type plasminogen activator (u-PA). The activity of plasminogen activators can be regulated by specific inhibitors of which at least three are known: plasminogen activator inhibitor type-1 (PAI-1), type-2 (PAI-2) and protease nexins (2,3). The inhibitors are thought to have a regulatory function in extracellular proteolytic processes.
Despite the importance of PAIs in the regulation of PA activity, it is still largely unknown which tissues and cells produce PAIs in vivo. In a previous study (4) we showed that coincubation of intact rat aorta with u-PA (or t-PA) results in a loss of PA activity, and in the formation of PA complexes with a molecular weight similar to that of PA-PAI complexes.
Extracts of total rat aorta contain both t-PA antigen and t-PA activity (5), which activity is reduced in aortas obtained from rats pretreated with endotoxin (6). In fibrin autographs, aorta extracts show two bands of PA activity, one of which is due to free t-PA, while the other is due to a t-PA-PAI complex (5). Taken together, these studies

suggest that in normal rat aorta both t-PA and a PA inhibitor are present, while endotoxin shifts the balance between these components to more inhibition.

The rat aorta is composed of three layers: intima, media and adventitia. Each of these three layers contains a homogenous cell population; the intima contains exclusively endothelial cells, the media contains only smooth muscle cells, while the adventitia contains fibroblasts and occasionally capillary endothelial cells (7,8,9). The three layers can be separated relatively easy by physical manipulation, without recourse to the use of proteolytic enzymes (10). This situation is particularly advantageous for the analysis of the in vivo mRNA composition of cells from this tissue, because the isolation of cells from blood vessels by e.g. proteolytic digestion may result in the rapid induction of some species of mRNA (11), which makes the extrapolation of such data to the in vivo situation hazardous. The rat aorta thus seems an ideal tissue to study the in vivo distribution of PA and PAI mRNA and their activities in pure cell populations.

Of additional interest might be that after injury vascular smooth muscle cells are involved in the process of vascular repair, resulting in the migration and proliferation of these cells. A recent report suggests that the PA content of vascular smooth muscle cells changes during vascular repair, u-PA being induced in proliferating vascular smooth muscle cells (12). From the fibrinolytic point of view these smooth muscle cells are also of interest, since previous histochemical studies have shown that the medial layer of blood vessels contain fibrinolysis inhibitory activity, while the intimal and adventitial layers show PA activity (13).

Moreover, several groups recently studied the production of plasminogen activators and inhibitors by smooth muscle cells in culture (14,15,16).

Endotoxin increases the level of PAI-1 mRNA in endothelial cells in vitro (17) as well as in the more vascularized tissues in vivo (6). PAI activity in cultured smooth muscle cells is also increased by endotoxin (unpublished observations).

It has not yet been established what the contribution of the different layers of the vessel wall is in the production of plasminogen activators and their inhibitors, nor how the different cell types of the vessel wall are involved. Whether the modulation of PA and PAI activity in the different cell types in vitro is comparable to modulation in vivo has, to our knowledge, not been demonstrated.

In the present study we describe the in vivo distribution of plasminogen activator and inhibitor production in the different layers of the aorta, and the modulation of the production by endotoxin treatment. Furthermore, we suggest that smooth muscle cells play an important role in the production of PAI-1 in vivo.

MATERIALS AND METHODS.

Male Wistar rats (280-320 g), obtained from the Broekman Institute, Someren, The Netherlands, were used. All animal experiments were performed under Nembutal (Sanofi, Paris, France) anaesthesia (60 mg/kg i.p.)

Endotoxin treatment

Rats were intravenously injected with endotoxin (Escherichia coli lipopolysaccharide, serotype 0128:B12, prepared by phenol extraction; Sigma, St.Louis, MO.) 50 μg/kg in a volume of 1 ml/kg. Three hours after the injection, just prior to the maximal plasma PAI activity (6), the blood and tissue samples were obtained. Control rats were injected with saline (1 ml/kg).

Aorta samples

Animals were bled by carotid cannulation. The aortas were then rapidly dissected and cleaned of connective tissue and fat. After a rinse with saline to eliminate adherent blood, the vessel was opened longitudinally and the media was completely dissected from the adventitial layer (10). In half of the aortas studied (control and endotoxin treated), the endothelium was removed by scraping with the blunt part of a scalpel. The presence of the endothelium on the untreated media and the successful removal of the endothelium from the scraped media was confirmed by scanning electron microscopy. Tissue samples were immediately frozen in liquid nitrogen and stored at -70°C till analysis. Samples for mRNA evaluation were processed directly.

Tissue extracts

Tissues were triturated in liquid nitrogen and homogenized (40 mg of wet weight/ml) in extraction buffer for 1 minute at 4°C by means of a Polytron-7 blender. After centrifuging the homogenate for 10 minutes at 3000 x g (4°C), the supernatant was filtered (Gelman Acrodisc filter, pore size 1.2 μM) and stored at -70°C (5). As extraction buffer, we used an acid acetate-buffer (75 mM acetic acid, 225 mM NaCL, 75 mM KCl, 10 mM EDTA, 100 mM arginine, 0.25 % Triton X-100, pH=4.2) (18). The concentration of tissue extract is expressed as mg of initial wet weight/ml.

Fibrin autography

Tissue extracts were subjected to SDS-PAGE (19) with a 9% separating gel and a 4% stacking gel. After electrophoresis, the gels were washed in 2.5% Triton X-100 and incubated on a plasminogen-rich fibrin-agarose underlay (20).

t-PA activity

PA activity in the tissue extracts was evaluated spectrophotometrically (21) in the presence and absence of anti-rat t-PA IgG (5). The difference between these activities was taken as t-PA activity. t-PA activity is expressed as Units, one U being defined as the amount of rat t-PA that gives the same rate of change in absorption in our assay as one international unit (IU) human t-PA (International Standard 83/517, National Institute for Biological Standards and Control, London, U.K.). t-PA activity was corrected considering the plasmin inhibitory activity of tissue extracts, as described (5).

PAI activity

A spectrophotometric assay was used to evaluate PAI activity (22), using human recombinant t-PA (Activase; Genentech, San Francisco, CA) to titrate PA inhibitory activity. No previous activation step was performed. Results are expressed in units. One U of rat PA inhibitory activity is that amount of PA inhibitory activity that quenches one IU of human t-PA.

t-PA antigen

t-PA antigen concentrations were determined using a commercial enzyme-linked immunoabsorbent assay (ELISA) (Imulyse, Biopool, Umea, Sweden). The monospecific antiserum used in this assay cross-reacts with rat t-PA but does not react with any other rat plasma protein (5). Rat t-PA purified from the yolk sac tumor cell line L2 (23) was used as standard. The t-PA antigen concentration is expressed in relation to its activity, one Unit of rat t-PA antigen being equivalent to one Unit of rat t-PA activity as defined above.

RNA isolation

Directly after removal from the rat, tissues were homogenized in 4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.5, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol. RNA was isolated from the homogenate according to Chomczynski and Sacchi (24). Total RNA was quantified by absorbance at 260 nm.

mRNA analysis

The following cDNA fragments were used as probes in the hybridization experiments: a 1.9 kb BglII fragment of the human t-PA cDNA (25), a 1.0 kb EcoRI PstI fragment of the human u-PA cDNA (26) kindly provided by Dr. W.D. Schleuning, a 0.9 kb PvuII fragment of the rat PAI-1 cDNA (27) kindly provided by Dr. T.D. Gelehrter, and a 1.2 kb PstI fragment of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (28) kindly provided by Dr. R. Offringa. The cDNA fragments were labeled with 32P-

dCTP (Amersham, United Kingdom) using a random primer method (Multiprime, Amersham, UK).

RNA samples were electrophoresed on a 1.2% agarose gel containing 7.5% formaldehyde. The RNAs were transferred to a nylon membrane (Hybond N, Amersham, UK) using a VACUGENE system (Pharmacia, Sweden). The membranes were hybridized with labeled cDNA fragments at 60°C in a solution containing 7% SDS, 0.5 M $NaHPO_4$ pH 7.2, 10 mM EDTA. The blots were routinely washed with 2xSSC, 1% SDS for 1 hr at 60°C (1xSSC = 0.15 M NaCl, 0.015 M sodium citrate).

Autoradiograms were prepared using KODAK XAR-5 film and intensifying screens at -70°C.

RESULTS

Endotoxin injection of rats (50 μg/kg) results in a strong decrease of t-PA activity in the aorta (71%) 3 hours after injection and an increase in plasma PA inhibitory activity, as described (6). In this study the changes in t-PA and PA inhibitory activity in the aorta were analyzed in further detail, using the same injection scheme.

PA activity.

Because of the small amount of vascular tissue, for each individual extract the aorta or aortic layers from two rats were pooled before extraction.

The plasminogen activator activities were first analyzed qualitatively by fibrin autography (fig 1). Uncomplexed (free) t-PA was observed both in adventitia and media layers, plus or minus intima. No band was found in the rat u-PA position, either in adventitia or in media extracts. Tissue extracts of aorta layers from endotoxin-treated rats showed in the t-PA position a smaller band than the extracts from control rats. All extracts showed a band in the t-PA:PAI complex position.

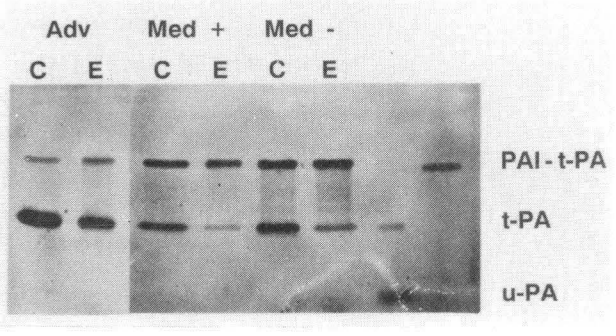

Figure 1. Fibrin autography of extracts from different layers of the aorta, adventitia (Adv), media plus endothelium (Med +) and media minus endothelium (Med -), of control (C) rats and endotoxin (E) treated rats. Endotoxin (50 μg/kg) was injected 3 hours before killing and tissue extraction. Equal quantities of sample for each tissue before and after endotoxin were used. Rat urine, containing t-PA and u-PA, and conditioned medium of cultured endothelial cells, containing t-PA/PAI complex, were used as standards.

Quantitative evaluation of plasminogen activator activity by spectrophotometric analysis (table 1) showed a detectable value in the adventitia layer only. In the media layer, plus or minus endothelium, no PA activity was detectable. PA activity was completely blocked when samples were evaluated in presence of anti-rat-t-PA. The t-PA activity in the adventitia was significantly ($p<0.01$) decreased by 75% in the endotoxin treated rats when compared to control rats (table 1).

TABLE 1

		Adventitia	Media (+/- Endothelium)
t-PA activity (U/g wet weight)	control	81.6 ± 44.5	n.d.
	endotoxin	20.5 ± 13.4	n.d.
	(n=6)	p<0.01	
t-PA antigen (U/g wet weight)	control	40.5 ± 20.0	40.9 ± 14.0
	endotoxin	36.9 ± 18.6	43.3 ± 14.1
	(n=6)		
PAI activity (U/g wet weight)	control	n.d.	192 ± 25
	endotoxin	n.d.	284 ± 41
	(n=5)		p<0.01

Tissue-type plasminogen activator (t-PA) activity and antigen, and plasminogen activator inhibitor (PAI) activity in different layers of the aorta of control rats and rats treated with endotoxin. Endotoxin (50 μg/kg) was injected 3 hrs before killing and isolation of the tissue. Activities and antigen concentrations were determined as described in materials and methods and expressed per g wet weight. Standard deviations are indicated. Non detecable levels are indicated by n.d.

t-PA antigen.

t-PA antigen was detected in extracts from both layers, media and adventitia, (table 1), with no significant differences between media plus and media minus endothelium. Thus, in table 1 the values of media plus and minus endothelium are combined. No differences in t-PA antigen were observed between control rats and endotoxin treated rats.

PA inhibitory activity

Spectrophotometric analysis of PA inhibitory activity showed that only in media extracts PAI activity could be detected (table 1). No significant differences were observed between media plus endothelium and media minus endothelium, 19.9 vs 20.3 U/mg protein and 29.8 vs 34.4 U/mg protein for control and endotoxin treated rats respectively. By endotoxin treatment PA inhibitory activity in the media was increased by 48% ($p<0.01$) (table 1). When PA inhibitory activity was evaluated in presence and absence of anti-rat-PAI-1 (29), PA inhibitory activity was decreased by 29% in the control group

and by 54% in the endotoxin treated group, resulting in a residual (non PAI-1 related) inhibitory activity that was similar for the control and the endotoxin-treated group.

mRNA analysis.

To determine whether the effect of endotoxin treatment on the PA inhibitory activity is due to an increased local synthesis of PAI-1 we determined the mRNA levels for PAI-1 in the total aorta of control rats and of endotoxin-treated rats. Endotoxin increased the mRNA levels for PAI-1 markedly (figure 2). To determine which part of the aorta wall was responsible for the PAI-1 production, RNA was isolated from the different layers of the aorta wall, the adventitia, the media plus endothelium and the media without endothelium. In control rats PAI-1 mRNA was detected mainly in the media, though a weak signal was also visible in the adventitia. After endotoxin treatment a strong increase of PAI-1 mRNA in the media was observed, while also in the adventitia PAI-1 mRNA now became clearly detectable (figure 2). No difference could be detected between media plus endothelium and media minus endothelium (figure 3).

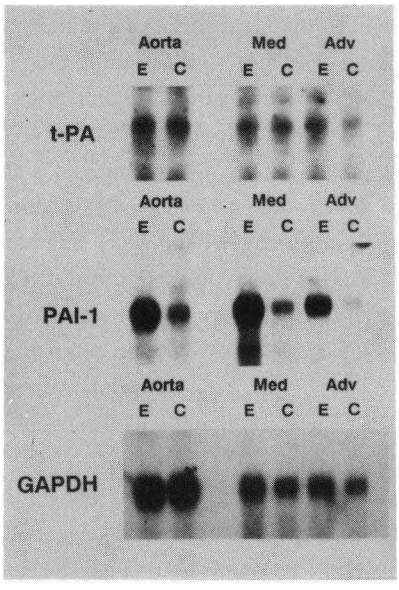

Figure 2. Northern blots of RNA extracted from total aorta or the different layers ot the aorta, adventitia (Adv) or media plus endothelium (Med), of control (C) rats and rats treated with endotoxin (E).
Endotoxin (50 μg/kg) was injected 3 hours before killing and RNA extraction. 10 μg total RNA was used in every lane. The filters were subsequently hybridized with DNA fragments of human t-PA, rat PAI-1 and rat GAPDH.

t-PA mRNA was detectable in the total aorta, and the mRNA level was not affected by the endotoxin treatment. Analysis of the t-PA mRNA levels in the different aorta layers showed that both in the media and in the adventitia comparable amounts of t-PA mRNA were present, and that the amount of t-PA mRNA was comparable to the amount of t-PA mRNA in the total aorta (figure 2). u-PA mRNA was neither detectable in the total aorta nor in any aorta layer, although, as shown previously, the human cDNA probe used does react with rat u-PA (6).

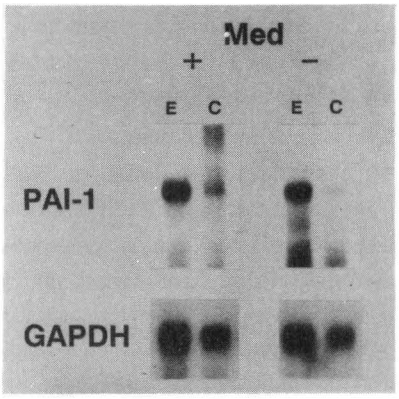

Figure 3. Northern blot of RNA extracted from the media plus endothelium (+) and the media minus endothelium (-) from the aorta of control rats (C) and rats treated with endotoxin (E). Endotoxin (50 μg/kg) was injected 3 hours before killing and RNA extraction. 10 μg total RNA was used in every lane. The filter was subsequently hybridized with cDNA fragments of rat PAI-1 and rat GAPDH.

DISCUSSION

In the present study we took advantage of the fact that the aorta can readily be separated into various layers, containing well-defined cell types, thus obviating the use of proteolytic enzymes to isolate cells, which might induce changes in cellular mRNA composition (11). In the media layer, which contains vascular smooth muscle cells only, we found mRNA for t-PA and PAI-1, t-PA antigen (an assay for rat PAI-1 antigen is not available) and, by spectrophotometric assay, PAI-1 activity but no t-PA activity. By fibrin autography we observed, next to free t-PA activity, a t-PA activity band which migrated at the position of t-PA:PAI complexes, and might thus be t-PA complexed to PAI-1. The difference between the spectrophotometric assay and fibrin autography as regards t-PA activity is likely due to the fact that extracts from the media layer contain complexes, which have no direct enzymatic activity, but will separate upon electrophoresis in Laemmli gels to produce a band of free t-PA activity. These complexes will also become active themselves upon electrophoresis in a Laemli system (30).

In previous studies Booth et al. (31) demonstrated the presence of PAI-1 antigen in various human tissue extracts, most pronouncedly in liver, kidney, lung and spleen. We have demonstrated that PAI-1 mRNA is present in most rat tissues (6), though we could not demonstrate PAI activity in tissue extracts (5). To our knowledge, this paper is the first to describe the presence of both PAI-1 mRNA and PAI-1 activity in a single cell type (the vascular smooth muscle cell) in vivo. Previously, we have shown (6) that rat liver endothelial cells contain PAI-1 mRNA. Assuming that the PAI-1 immunoreactive material described by Eriksen (32) in adrenal medullary cells represents locally synthesized material, then three cells are known now to produce in vivo PAI-1: liver endothelial cells (6), vascular smooth muscle cells (this paper) and medullary adrenal

cells (32). Our observations do not shed further light on the question whether vascular endothelial cells synthesize PAI-1 in vivo, as extracts of media with and without endothelial cells proved indistinguishable in all our assays. Presumably, due to the much larger tissue mass of the smooth muscle cells compared to the endothelial cell mass, the smooth muscle cell-related effects superseded any endothelial cell-related effect.

Of note is that, of the PA inhibitory activity in the medial layer extract, only some 30% was due to PAI-1. Which inhibitor(s) accounted for the remaining two-thirds of the medial inhibitory activity is not known. In culture, vascular smooth muscle cells have been shown to produce three types of inhibitory activity: PAI-1, PAI-2 and protease nexin I (14).

The presence of excess t-PA activity in the adventitia would agree with previous histochemical observations on the distribution of plasminogen activator activity in aorta (33), where activity has mainly been found in relation to the endothelium of the intima, and in relation to capillary vessels (if present) in the adventitia. Which cell in the adventitia is responsible for the synthesis of t-PA can not be deduced from our data, as a few capillary endothelial cells may have been present in our adventitias. Fibroblasts are, however, a likely source in view of their predominance.

After endotoxin treatment, t-PA activity in the adventitia was decreased by 75%. The t-PA antigen and mRNA levels in the adventitia and media were, however, not affected by endotoxin treatment, whereas the PAI-1 mRNA levels as well as PA inhibitory activity in the media were increased. This indicates that the change in the t-PA activity is likely due to an increase of plasminogen activation inhibition. Which cells in the adventitia are responsible for the PAI-1 mRNA production after endotoxin treatment can not be deduced from our data for the reasons discussed.

Addition of specific antibodies against rat PAI-1 to extracts of endotoxin-treated or control media, reduced the inhibitory activity to a comparable residual level, indicating that the endotoxin induced increase in plasminogen activator inhibitory activity is due to an increase in PAI-1. After endotoxin treatment PAI-1 mRNA was increased in the media and became also clearly detectable in the adventitia. No difference between media plus endothelium and media minus endothelium could be detected. The role of smooth muscle cells as compared to vascular endothelial cells in the PAI-1 production is especially of interest if the estimated weights of smooth muscle cells and endothelial cells, 2 kg and 270 g respectively in a 70 kg man (34), are taken into account. When secreted into the circulation, PAI-1 may play a role in the regulation of fibrinolysis, whereas when retained in the vessel wall, probably in complex with vitronectin embedded in the extracellular matrix (16,35), it might be implicated in regulation of local proteolytic events associated with eg. cell migration.

It has been reported that PAI-1 levels in vivo as well as in vitro can be modulated by various agents (for review see: 36), including endotoxin (6,17,37,38). The fast and dose-

dependent increase in plasma PAI activity after endotoxin injection is regulated at the mRNA level (6). It has been suggested that endothelial cells were mainly responsible for the increase in PAI-1 mRNA after endotoxin treatment, since in in vitro cultures of endothelial cells a rapid induction of PAI-1 mRNA by endotoxin is observed (17) and since the more vascularized tissues reacted more strongly. It was also shown that, in the liver, the sinusoidal endothelial cells showed the most prominent effect of endotoxin on the PAI-1 mRNA levels (6). In the aorta, however, no or only very little difference in PAI-1 mRNA could be detected in media with or without endothelial cells, indicating that smooth muscle cells contribute significantly to the PAI-1 production. It can not be concluded, however, from our data, whether endothelial cells are minor producers of PAI-1 since they form only a small fraction of the cells in the media plus intima layer. It would be interesting to know whether the effect of epidermal growth factor and transforming growth factor-β on the production of PAI-1 by the aorta, as reported recently by Fujii et al. (39) is caused by an increase in the endothelium or in the medial smooth muscle cells.

The production of plasminogen activators and their inhibitors by vascular smooth muscle cells and adventitia tissue might indicate that besides the well documented role of the plasminogen activator system in the circulation an equally important role in local proteolytic processes, like cell migration, within the vessel wall should be considered.

Note added in proof:

Immunohistochemical staining of the rat aorta with antibodies against rat PAI-1 showed that in the smooth muscle cell layer a strong signal was detectable, while in the endothelium only a weak signal could be detected (personal communication Dr. P. Roholl).

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CHAPTER 4

PROTEIN AND mRNA LEVELS OF PLASMINOGEN ACTIVATORS AND INHIBITORS ANALYZED IN 22 HUMAN TUMOR CELL LINES

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Cancer Research (1990) 50; 1488-1494

ABSTRACT

In 22 human tumor cell lines the regulation of production of plasminogen activators, urokinase (u-PA) and tissue-type (t-PA), and their inhibitors PAI-1 and PAI-2, was studied. These four components may determine the net plasminogen activator activity, which is often associated with tumor development and metastatic processes. The amount of specific mRNA and protein produced by the cells was measured for all four components.

The frequent finding of t-PA (alone or in combination with u-PA) suggests that t-PA can also be a tumor-associated plasminogen activator. In 11 of the 22 cells PAI-1 mRNA and in 6 of the 22 cells PAI-2 mRNA was found, pointing to a possible role of plasminogen activator inhibitors in the tumor related plasminogen activator activity.

This study demonstrates that there are at least two important regulatory steps in the regulation of production of plasminogen activators and their inhibitors. First, the regulation at the mRNA level, since a high protein amount is always correlated with a high mRNA amount found in the tumor cells. Secondly, there must be a significant regulatory step at the (post)translational level as can be concluded from differences in mRNA usage.

INTRODUCTION

Plasminogen activators are serine proteases that can activate the zymogen plasminogen to the active serine protease plasmin. Two types of plasminogen activators have been described, tissue-type plasminogen activator (t-PA),
and urokinase-type plasminogen activator (u-PA). They are encoded by different genes and differ immunologically and in enzymatic characteristics (1-8).

The activity of plasminogen activators can be inhibited by specific inhibitors for plasminogen activators (for review: 9,10). At least two specific inhibitors have been described, plasminogen activator inhibitor type 1 (PAI-1) (11,12) and type 2 (PAI-2) (13-18).

Plasminogen activators may play an important role in a variety of proteolytic events, such as fibrinolysis, tissue-remodelling, pericellular proteolysis and malignancy (19,20). Several hypotheses have been proposed concerning the role of plasminogen activators in the metastatic process (19-23). First, plasminogen activators have been implicated in the first step of metastasis, the breakdown of the basal membrane to enable the cells to detach from the primary tumor and reach the circulation (19-21). Secondly, within the circulation, tumor cells often acquire a fibrin coat (24-26) which is thought to be involved in evading the immune response and immobilization of tumor cells in the microvascular system. Plasminogen activators and their inhibitors can clearly be involved in the

formation and breakdown of this fibrin coat. Thirdly, plasminogen activators are thought to mediate the proteolytic breakdown of the extracellular matrix at the site of tissue invasion, preceding the formation of a seco ary tumor (19-23). Lastly, plasminogen activators may have a function in the process of angiogenesis, i.e. the formation of new blood vessels supplying the newly formed tumor with nutrients required for rapid growth (27,28).

Despite the fact that an increased plasminogen activator activity has been generally found in many tumor tissues (20) and tumor cells in culture (19,20, 29,30,31) when compared with normal controls, only in a few cases has the actual involvement of plasminogen activators in the metastatic process been shown (32). Although the exact function of plasminogen activator activity is not completely understood, it has been successfully used as a marker for the diagnosis of malignant transformation (33,34,35). These changes of plasminogen activator activity may be due to altered levels of u-PA or t-PA or of their inhibitors, PAI-1 or PAI-2. Comparative studies of the t-PA and u-PA protein levels, as well as the PAI-1 protein level in human tumor cells have been described (30,36).

To investigate the importance of the various steps in the regulation of plasminogen activator activity in tumor cells, regulation at the mRNA level and at the level of translation efficiency or posttranslational events, we have determined both the mRNA levels and the protein levels of the plasminogen activators, u-PA and t-PA, and the plasminogen activator inhibitors, PAI-1 and PAI-2, in twenty-two human cell lines from a variety of malignancies.

MATERIALS AND METHODS

Cell lines

All the cell lines are derived from human origin. The Bowes melanoma, fibrosarcoma HT1080 and endothelial hybridoma Edgell cell line (37) were obtained from Dr. J.J. Emeis (Gaubius Institute TNO, Leiden, The Netherlands), the uterine cervix carcinoma HeLa S3 and its subline HeLa NIBSC from Dr. J.F. Cajot (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland), the breast carcinoma MCF7 from Dr. K. Hoekman (University Hospital, Leiden, The Netherlands), the epidermoid carcinoma A431 from Dr. R. Offringa (Sylvius Laboratory, Leiden, The Netherlands), the hepatoma Hep G2 from Dr. H.M.G. Princen (Gaubius Institute TNO, Leiden, The Netherlands); the histoic lymphoma U937 and the promyelocytic leukemia HL60 from Dr. H.J. Kempen (Gaubius Institute TNO, Leiden, The Netherlands).

The other cell lines used are the colon carcinoma SW948, SW707, SW2219, HT29, CaCo2, Colo205, SW480, SW620, a lymph node metastasis of SW480, the gastric carcinomas HGT1 (38) and KATO III, the pancreas carcinoma HS766T, and the myelo-

genous leukemia K562. All cells were mycoplasma-free when tested according to Russel et al. (39).

Cell culture

Cell lines were grown in the following culture media, supplemented with 20 mM HEPES, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin: Bowes, HT1080, Edgell, HeLa S3, HeLa NIBSC, MCF7, HS766T, HT29, HGT1, Hep G2 in DMEM and 10% FCS. SW480, SW620, SW707, SW948, SW2219, A431, Colo205, in RPMI 1640 and 10% FCS. CaCo2 and KATO III in RPMI and 20% FCS. The non-adherent cells K562, U937, HL60 were grown to a density of ± 0.5 x 10^6 cells per ml in RPMI 1640 and 10% FCS (K562 and U937) or 20% FCS (HL60) before the experiment started. Just prior to confluency, fresh medium (as described above) was added and twenty-four hours later when the cells had reached confluency, the conditioned medium was collected and from a number of parallel cultures cell extracts were made for the purpose of antigen determination, DNA determination and RNA isolation.

Determination of DNA content

For the DNA determination from at least two parallel cultures of each cell line extracts (extracted with H_2O) were collected. The DNA content was measured as described by Brunk et al. (40), using 4,6,-diamidino-2-phenylindole (DAPI) as a fluorescent reagent. Because determination of the cell numbers was difficult for some of the cell lines, all data were expressed per μg DNA. The DNA content per cell, as far as it could be determined, varied between 5-12 pg per cell.

Assay of PA and PAI antigen

The antigens were determined in duplicate both in conditioned media and cell extracts (extracted with 0.5% Triton X-100). u-PA antigen was determined with a sandwich ELISA described in detail by Binnema et al. (41). t-PA antigen was determined using the commercially available ELISA Imulyse t-PA (Biopool, Umeå, Sweden). PAI-1 and PAI-2 antigen were determined using the commercially available ELISA Tintelize PAI-1 and Tintelize PAI-2 (Biopool, Umeå, Sweden). The detection limits for these assays are approximately 0.5 ng for u-PA and t-PA, 5 ng for PAI-1 and 0.1 ng for PAI-2 per assay volume. In all cases blank determinations of non-conditioned but serum-containing medium were done and subtracted from the results obtained with conditioned media. These corrections were very small however.

RNA isolation

Cells were washed with PBS at 37 °C and lysed in buffer (100 mM NaCl, 7.5 mM EDTA, 50 mM Tris.HCl, pH 7.4, 0.5% SDS, proteinase K (150 μg/μl)) during 30 min at 37 °C.

After lysis the cell extracts were homogenized for 30 sec with a polytron homogenizer. The cell homogenates were extracted twice with one volume of phenol:chloroform:isoamylalcohol (50:48:2) and once with one volume of chloroform:isoamylalcohol (96:4). RNA was obtained by overnight precipitation with 3 volumes of 3 M LiCl at 0°C, followed by precipitation with ethanol (42,43).
The total amount of RNA isolated was determined by measuring the A260, assuming that 1 A260 unit was 40 μg RNA. For each total RNA isolated the A260 A280 ratio was determined and an agarose gel was run and stained with ethidium bromide. From these experiments we concluded the contamination with DNA was negligible. Part of the total RNA isolated was poly A^+ selected using oligo dT-cellulose (type 77T, Pharmacia, Woerden, The Netherlands). From all cell lines the poly A^+ RNA was used for denaturating agarose electrophoresis and blotted to nylon filters (Hybond N., Amersham, Houten, The Netherlands) (Northern blotting). Filters were hybridized with the different probes and visualized with autoradiography.

Probes

The following cDNA fragments were used as probes in the hybridization experiments: a 1.9 kb BglII fragment of the human t-PA cDNA (44) a 1.0 kb EcoRI-PstI fragment of a human u-PA cDNA (45), a 2.5 kb EcoRI fragment of a human PAI-1 cDNA of the 3.1 kb transcript (43), a 1.2 kb EcoRI fragment of a human PAI-2 cDNA (provided by Dr. E.K.O. Kruithof, 46), a 1.4 kb NcoI BglII fragment of the human fibrinogen Aα chain (provided by Dr. S. Lord, 47), and a 1.2 kb PstI fragment of a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA provided by Dr. R. Offringa (48), which is commonly used as an internal standard probe (49). The cDNA fragments were labeled radioactively using the random primer method (Multiprime, Amersham, Houten, The Netherlands), with
^{32p} dCTP.

In vitro transcripts

The t-PA 1.9 kb BglII cDNA fragment, u-PA 1.0 kb EcoRI PstI cDNA fragment, PAI-1 2.5 kb cDNA fragment and PAI-2 0.9 kb PstI EcoRI cDNA fragment were cloned into the transcription plasmids pGEM3Z or pGEM4Z (Promega, Leiden, The Netherlands). After linearization with the appropriate restriction enzymes these plasmids were used for in vitro transcription (50) with T7 RNA polymerase according to the manufacturers instructions. After transcription the DNA templates were removed using RNAse-free RQ1 DNAse.
The amount of RNA transcripts synthesized was determined by measuring the A260. These transcripts were used as standards in the quantitative hybridization experiments.

Table 1. Protein production and mRNA level in different tumor cell lines.

A. Results for u-PA and t-PA.
Column 1: cell type and origin. Column 2 and 5: sum of u-PA and t-PA production respectively in medium and cell extracts expressed per μg DNA. Specific protein was determined by ELISA and DNA by fluorescent staining (see Materials and Methods). Column 3 and 6: total amount of specific mRNA for u-PA and t-PA respectively, expressed per μg DNA, as determined using dot blot hybridization (for details see figure 2 and Materials and Methods). Column 4 and 7: u-PA and t-PA respectively in the medium expressed as percentage of the total amounts in the medium plus the cell extracts. Amounts below detection limit are indicated by dashes.

		A. u-PA and t-PA					
		u-PA			t-PA		
1		2	3	4	5	6	7
Cell type and origin		ng u-PA /μg DNA	pg mRNA /μg DNA	% u-PA in medium	ng t-PA /μg DNA	pg mRNA /μg DNA	% t-PA in medium
Bowes	Melanoma	-	-	-	68	118	96
HT1080	Fibrosarcoma	16	51	88	39	119	91
Edgell	Hybridoma	4.3	33	35	32	38	96
HeLa NIBSC	Cervix carcinoma	-	-	-	0.6	0.8	60
HeLa S3	Cervix carcinoma	-	-	-	1.4	1.6	83
MCF7	Breast carcinoma	0.1	0.04	100	0.2	0.11	24
A431	Epidermoid carcinoma	83	28	94	2.7	2.3	67
K562	Myelogenous leukemia	0.7	0.2	100	0.8	0.2	52
Colo 205	Colon carcinoma	1.3	0.3	70	0.8	0.2	52
KATOIII	Gastric carcinoma	2	-	88	0.2	-	0
CaCo2	Colon carcinoma	0.8	0.2	80	-	-	-
HT29	Colon carcinoma	0.3	0.2	50	0.2	-	40
HGT1	Gastric carcinoma	0.3	-	100	0.2	-	37
HepG2	Hepatoma	0.3	-	71	-	-	-
SW620	Lymph node metastasis of SW480	1	2.1	44	0.2	2.4	37
SW480	Colon carcinoma	0.8	8.7	48	0.5	6	40
SW2219	Colon carcinoma	0.3	0.14	70	0.4	0.2	39
SW948	Colon carcinoma	0.3	-	100	0.4	-	41
SW707	Colon carcinoma	0.1	0.3	100	0.4	0.4	55
U937	Histoic lymphoma	0.9	0.2	36	0.2	-	47
HL60	Promyelocytic leukemia	5.5	1.9	25	0.9	-	100
HS766T	Pancreas carcinoma	0.2	0.3	0	1.6	25	76

Table 1. Protein production and mRNA level in different tumor cell lines.

B. Results for PAI-1 and PAI-2.

		B. PAI-1 and PAI-2					
		PAI-1			PAI-2		
1		2	3	4	5	6	7
Cell type and origin		ng PAI-1 /µg DNA	pg mRNA /µg DNA	% PAI-1 in medium	ng PAI-2 /µg DNA	pg mRNA /µg DNA	% PAI-2 in medium
Bowes	Melanoma	-	-	-	1.6	103	0
HT1080	Fibrosarcoma	71	585	88	2.4	26	0
Edgell	Hybridoma	492	3400	97	0.2	17	33
HeLa NIBSC	Cervix carcinoma	0.8	33	100	-	-	-
HeLa S3	Cervix carcinoma	0.3	8.6	100	0.1	19	67
MCF7	Breast carcinoma	0.1	-	100	-	-	-
A431	Epidermoid carcinoma	2.4	366	80	9	89	30
K562	Myelogenous leukemia	-	-	-	-	-	-
Colo 205	colon carcinoma	-	-	-	-	-	-
KATOIII	Gastric carcinoma	0.3	1.6	100	-	-	-
CaCo2	Colon carcinoma	0.3	-	100	-	-	-
HT29	Colon carcinoma	0.5	-	73	-	-	-
HGT1	Gastric carcinoma	-	-	-	-	-	-
HepG2	Hepatoma	10	3.9	66	-	-	-
SW620	Lymph node metastasis of SW480	1.9	16	78	-	-	-
SW480	Colon carcinoma	16	945	87	-	-	-
SW2219	Colon carcinoma	3.9	2.2	96	-	-	-
SW948	Colon carcinoma	-	-	-	-	-	-
SW707	Colon carcinoma	-	-	-	-	-	-
U937	Histoic lymphoma	-	-	-	-	-	-
HL60	Promyelocytic leukemia	-	-	-	2.3	15	11
HS766T	Pancreas carcinoma	0.2	3.9	100	-	-	-

RESULTS

Quantitative analysis of mRNA

Since no specific hybridization signal could be detected when total cellular RNA from most of the investigated cells was applied on Northern blots, experiments were performed with poly A^+ selected RNA.

It was found that all probes hybridized with discrete bands with the expected length, indicating that the RNA's were intact and that hybridization was specific (shown for some representative cells in Fig. 1). For PAI-1 the previously described two mRNA's were seen (43) (Fig. 1). As an internal standard the GAPDH probe was used, since this gene is almost equally expressed in all cells (Fig. 1). The frequency of occurrence for the specific mRNA's is shown in table 2a,b.

When a positive signal was detected on a Northern blot, the RNA was quantitatively analyzed on a dot blot. For each RNA a series of dilutions was spotted on a filter.

Each of these filters also contained a series of dilutions of the four in vitro transcripts of u-PA, t-PA, PAI-1 and PAI-2 as standards. After hybridization and autoradiography, the amount of specific RNA present in each sample was estimated by comparison to the corresponding standards (Fig. 2). The detection limit of specific RNA which was detectable on Northern blot was ~ 0.01 pg RNA. On all dot blots reference lines for t-PA, u-PA, PAI-1 and PAI-2 transcripts were present. In no case any hybridization of a probe with another cognate RNA was observed, indicating that no cross hybridization occurred.

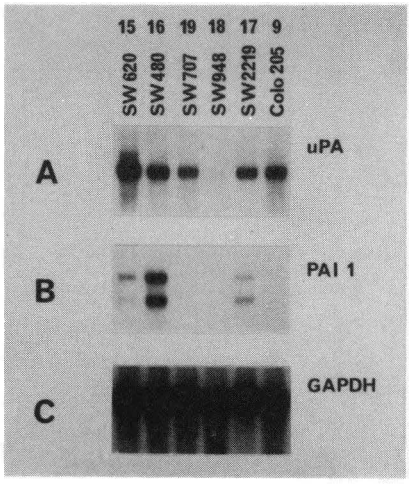

Figure 1. Qualitative mRNA analysis.
Poly A^+ RNA isolated from the different cell lines as described in Materials and Methods was subjected to electrophoresis and blotted. The blots were hybridized with specific probes and a GAPDH probe as a control. Amounts of poly A^+ RNA corresponding to 100 μg total RNA were used. This was done for all cell lines and all probes. In this figure only the results for u-PA (A) and PAI-1 (B) control GAPDH (C) for 6 representative cells of the 22 are shown. The numbers correspond to the cell lines in Table 1.

The same filters were consecutively hybridized for the four probes and GAPDH as an internal standard. The amounts of specific mRNA found were expressed per μg cellular DNA (Table 1a and b). These amounts were found to be very different for each cell type. Furthermore, the range of the specific mRNA found for PAI-1 was found to differ widely from that of the other mRNA's, of t-PA, u-PA and PAI-2. To investigate whether these differences were caused by variations in the methods, we have tested the reproducibility of the mRNA amount in four selected cells, Bowes, HT1080, A431 and HGT1 cells. For each cell four cultures were analyzed for t-PA, u-PA, PAI-1 and PAI-2 mRNA. The reproducibility within each cell line for each component was determined and was between 14 and 25% (SD, n = 4).

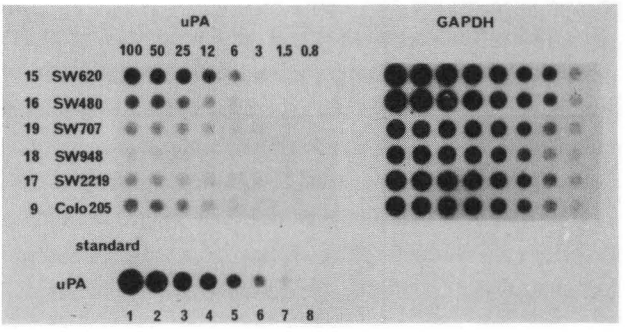

Figure 2. Quantitative mRNA analysis.
Serial dilutions of poly A^+ RNAs were spotted on the filter, hybridized with the different probes and visualized by autoradiography. Each filter contained standard dilution curves of RNA transcribed in vitro for u-PA, t-PA, PAI-1 and PAI-2. Quantification was performed by visually comparing as many points from the sample dilutions with the standard dilutions.
In the left panel an example is shown for u-PA in the same cell lines shown in figure 1. The corresponding amounts of total RNA's are indicated. Spots 1-8 of the standard line correspond with 1.15 ng, 0.58 ng, 0.29 ng, 0.15 ng, 0.08 ng, 0.04 ng, 0.02 ng, 0.01 ng u-PA mRNA.
In the right panel the filter is hybridized with GAPDH as a control.

Quantitative analysis of antigens

The levels of the respective proteins were determined immunologically. This enables us to compare directly the final gene product with the particular mRNA. We have used ELISA assays which are known to detect all forms (active, latent, complexed) of the respective proteins. Both the antigen levels in culture media and cell extracts were determined. The total amount per μg DNA is shown in Table 1a,b. Here also a great variation in detected amounts of protein was found. Again the reproducibility was tested with four parallel cultures for the same cell lines and components. In this case the reproducibility was between 6 and 24% (SD, n = 4). The frequency of occurrence for the specific proteins is shown in table 2a,b.

Protein secretion into the medium

The distribution of the plasminogen activators and the inhibitors between the medium and the cell extracts is shown in table 1a,b by the percentage of protein present in the culture media. The fraction of the respective proteins in the media varied for the different cells with the exception of PAI-1, for which in all cases 70% or more of the protein was found in the medium, when present.

Ratio of specific protein and mRNA

For an easier interpretation at the mRNA levels and protein levels, the ratio of protein versus mRNA was calculated for each cell line and each component (Fig. 3). This ratio represents the efficiency of protein production per mRNA. The efficiency of PAI-2 production in all cells investigated is in general considerably lower than that of u-PA, t-PA and PAI-1 (see Fig. 3, note scale differences). For Bowes, HT-1080, A-431 and HGT-1 cells the reproducibility of this ratio was determined in four parallel cultures of each cell as described for both mRNA and antigen before. It was found that within a cell line the variation between parallel cultures was between 10% for HT-1080 and PAI-2 and 33% for Bowes and t-PA. This indicates that differences in these ratios larger than 33% are likely due to differences between the cell lines. Most differences observed are much larger than this.

The efficiency of production of each protein in different cells is highly variable (see Fig. 3).

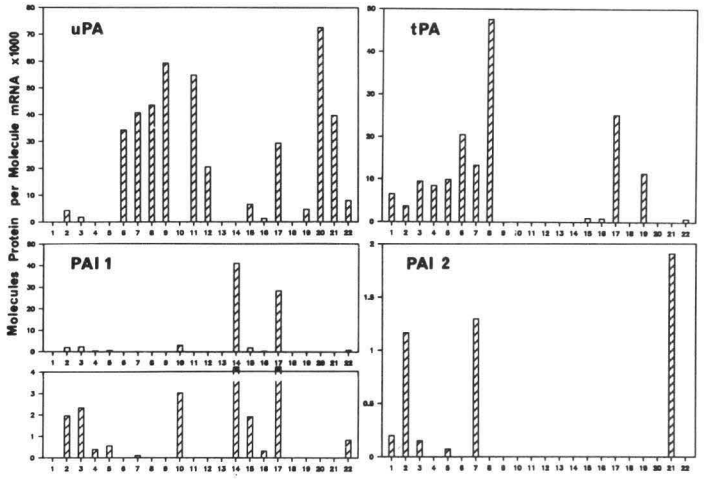

Figure 3. Ratio protein to mRNA. Ratio protein to mRNA as determined from Table 1a,b, column 2,5 and 3,6. The numbers along the horizontal axis correspond with the numbers of the cell lines in table 1. Along the vertical axis the protein mRNA ratio is expressed as molecules protein per mRNA molecule. The number of molecular mRNA was calculated using the average Mr of 321 per nucleotide and the transcript length. The number of protein molecules was calculated using the known Mr of these proteins. Note the different scales.

DISCUSSION

In the present paper some aspects of the regulation of plasminogen activation are described, i.e. the relationship between mRNA level and protein level of the plasminogen activators u-PA and t-PA and their inhibitors PAI-1 and PAI-2.
In all of the cells studied, at least one of the two plasminogen activators was detected (table 2a), although the quantities varied widely (table 1a). It is surprising that in this

Table 2. Frequency of plasminogen activator and inhibitor production in tumor cells.

a. Results for u-PA and t-PA
The number of tumor cells producing detectable u-PA or t-PA mRNA and protein, as well as the cells producing both or none, is shown.

	u-PA	t-PA	u-PA + t-PA	none
mRNA	15	13	10	4
protein	19	20	17	0

b. Results for PAI-1 and PAI-2
The number of cells producing detectable PAI-1 or PAI-2 mRNA and protein.

	PAI-1	PAI-2	PAI-1 + PAI-2	none
mRNA	11	6	4	9
protein	14	6	4	6

collection of tumor cells not only u-PA, which was known to be the tumor-associated plasminogen activator (19,20,23), but also t-PA was frequently found in comparable quantities (table 1a).
The phenomenon, however, that not in all the cell lines the corresponding mRNA was detected, might be due to differences in detection limit between mRNA and protein.
Since it is the activity of plasminogen activators that is thought to be involved in tumor development, it is not unlikely that plasminogen activator inhibitors play a role together with the enzymes. We have therefore performed similar investigations for the two inhibitors PAI-1 and PAI-2. In nearly all of the cells, either or both of these two types of inhibitors was found, but again the quantities were very different (table 1b,2b). This

supports the idea that for the activity of plasminogen activators in tumor development, the inhibitors should be studied as well as the enzymes.
It is striking that the cells which have the highest level of plasminogen activators, u-PA, t-PA or both (Bowes, HT1080, Edgell, A431, HeLa S3), also produce the highest levels of inhibitors, PAI-1, PAI-2 or both, suggesting a relation between the production of plasminogen activator and an inhibitor. The presence of PAI-2 in Bowes melanoma, the cell mostly used for t-PA production (51,52), is surprising.
The levels of all components studied were found to be highly different from cell line to cell line. To ensure that all cell lines were in a proliferative phase we have analyzed them in the last 24 h before reaching confluency. This has been chosen arbitrarily because in this way it is relatively easy to compare different cells with each other.
For a number of selected cell lines four parallel cultures were compared and it was found that the antigen levels varied between 6 and 24%, while for the mRNAs this variation was 14-25%. If we assume that these numbers are similar for the other cell lines used this means that variations beyond this range can be ascribed to differences between the cell lines and not between the cultures. The level of the protein is dependent on the level of the particular mRNA, the translation efficiency and possible posttranslational modifications. In the present study the relationship between the level of the particular mRNA and the corresponding protein was studied.
In general, it was seen that when a high protein level was found, a high level of the corresponding mRNA was also detected (Table 1). This points to an important role of regulation of the level of mRNA in the regulation of the production of the particular protein. The regulation of the level of mRNA can be accomplished by two different processes, namely, the transcription of the gene and the stability of the produced mRNA.

When Table 1 is studied in more detail, it can be seen that in some cases similar mRNA levels are associated with widely different protein levels. This suggests that the efficiency of mRNA usage differs significantly from cell to cell. In Fig. 3 the ratios protein to mRNA are shown for all components, visualizing more clearly the differences in mRNA usage between the cells. For u-PA, t-PA and PAI-2 the ratio differed by 10- to 25-fold, even when the mRNA levels were comparable. For PAI-1 the differences in ratio were even more pronounced, in A431 and Hep G2 the ratio differs by a factor 400. These ratios differ much more than can be ascribed to variations in cultures which were found to be only 10-33%. These results indicate that besides regulation of the mRNA level significant regulation also occurs at the (post)translational level.
For the proteolytic events associated with tumor development it is thought that extracellular plasminogen activator activity is required. Therefore, not only were the total amounts of plasminogen activator and their inhibitors determined, but also the amounts which were cell associated and which were found in the culture medium (see Table 1a,b).

It is shown that the percentage of the protein present in the culture medium can differ considerably. The recent discovery of a receptor for u-PA (5,6) and the finding that receptor-bound u-PA can retain its activity, might indicate that cell associated, rather than free plasminogen activators, may have a function in local proteolytic events in tumor development (19,20,53).

Some tumor cells are surrounded by a fibrin coat. This fibrin could have a function in metastasis of tumor growth (19,54). For this reason, the presence of fibrinogen mRNA in the 22 tumor cells was investigated. Only in two cases, the colon cell line CaCo2 and the hepatoma cell line Hep G2, was fibrinogen mRNA found (data not shown). From this it can be concluded that tumor cells in general do not produce fibrinogen themselves, but possibly use the abundant fibrinogen in the plasma.

The different regulation steps, mRNA level, (post)translational level, protein secretion, protein activation, especially of pro-u-PA and latent PAI-1, interaction with inhibitors and receptors, have their effects of the plasminogen activation activity on a different time scale. The interaction with the inhibitors and receptor is extremely rapid (10,11,12). Activation of proenzymes and the secretion of enzymes operate at an intermediate rate, while changes in the mRNA level are usually slow. A high mRNA level may give the cell the opportunity to quickly increase the production of the corresponding protein. The interaction of u-PA with its receptor and of PAI-1 with the extracellular matrix may highly localize their effect. These different steps of regulation of the plasminogen activator activity enables the tumor cells to fine tune this activity during the different stages of metastasis and tumor development.

From this study, it can be concluded that at least two regulation steps are operational, at the mRNA level and at a (post)translational level. It should thus be considered that the mRNA level alone does not give the full information, but that the actual protein production should also be taken into account. For tumor-associated plasminogen activator activity, not only should u-PA be considered but also t-PA and certainly PAI-1 and PAI-2, since the net plasminogen activator activity is determined by the balance of the activators and inhibitors. The recently discovered u-PA receptor may be even more important for these highly localized tumor-associated proteolytic processes.

Acknowledgements:

The authors wish to thank Dr. J.F. Cajot for providing us the HeLa S3 and HeLa NIBSC cells, Dr. K. Hoekman for providing the MCF7 cells, Dr. R. Offringa for providing the A431 cells and the rat GAPDH cDNA probe, Dr. E.K.O. Kruithof for the human PAI-2 cDNA probe and Dr. S.H. Lord for the fibrinogen cDNA probe.

We would like to thank also Petra Turion and Gerard Dooijewaard for their assistance with the u-PA antigen assays.

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CHAPTER 5

METASTATIC BEHAVIOUR OF HUMAN MELANOMA CELL LINES IN NUDE MICE CORRELATES WITH UROKINASE-TYPE PLASMINOGEN ACTIVATOR, ITS TYPE-1 INHIBITOR AND UROKINASE MEDIATED MATRIX DEGRADATION

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Submitted for publication

ABSTRACT

Five out of six human melanoma cell lines tested were able to degrade in vitro a smooth muscle cell extracellular matrix in a plasmin dependent way. In three of these five cell lines this process was mediated by tissue-type plasminogen activator (t-PA) and in the other two cell lines by urokinase-type plasminogen activator (u-PA).

All melanoma cell lines produced t-PA mRNA and protein, while only the two cell lines showing u-PA mediated matrix degradation produced u-PA mRNA and protein. These latter cell lines also produced plasminogen activator inhibitor type-1 (PAI-1) and type-2 (PAI-2) mRNA and protein. u-PA receptor (u-PA-R) mRNA and binding of radiolabeled u-PA was found in all melanoma cell lines.

The metastatic capacity of these cell lines was studied in nude mice. All cell lines were able to develop primary tumors at the subcutaneous inoculation site. The production of plasminogen activators, their inhibitors and urokinase receptor by subcutaneous tumors corresponded with the production by the parental cell lines in vitro.

The two u-PA and PAI-1 producing cell lines showed the highest frequency to form spontaneous lung metastases after subcutaneous inoculation, while 5 of the 6 cell lines formed lung colonies after intravenous inoculation.

In conclusion, u-PA mediated matrix degradation in vitro and production of u-PA and PAI-1 by human melanoma cell lines correlated with their ability to form spontaneous lung metastasis in nude mice. No correlation was found with the ability to form lung colonies after intravenous injection. These findings suggest a role for u-PA and PAI-1 in a relatively early stage of melanoma metastasis.

INTRODUCTION

During metastasis tumor cells must penetrate basement membranes and interstitial tissues, when they detach from the primary tumor and intravasate into the circulation, and later when they extravasate at the site formation of the secondary tumor. This means that these tumor cells should express the right panel and adequate levels of proteolytic enzymes to degrade the extracellular matrix (15,16,24,34,49,57). In addition, these enzymes may have a function in the process of angiogenesis when endothelial cells grow invasively into the newly formed tumor and form new blood vessels (25,38).

The serine protease plasmin is one of the major enzymes believed to be involved in such proteolytic processes (15,16,24,42,43). Plasmin has a broad substrate specificity and can digest most of the components of the extracellular matrix including the basement membrane, either directly or by activation of proenzymes of metalloproteinases, like type IV collagenase or interstitial collagenase (23,39,57). Plasmin is formed by a conversion

of the zymogen plasminogen, which is regulated by plasminogen activators. Two distinct plasminogen activators are known, the tissue-type (t-PA), and the urokinase-type (u-PA). The activity of the activators can be regulated by interactions with specific inhibitors, of which two have been described, type 1 (PAI-1) and type 2 (PAI-2) (33,53). In addition, u-PA and its proenzyme, pro-u-PA, can be localized at cell surfaces by binding through their growth factor domain to a specific receptor (u-PAR) (2,8,12,41,51,55,56,58).

In order to study the role of plasminogen activation system in metastasis of malignant melanomas, we have investigated the occurence of its various components and their mRNA in a set of six human melanoma cell lines with different metastatic behaviour in the nude mice. In order to study the different steps in the metastatic process (primary tumor growth, local invasion and detachment of tumor cells from the primary tumor, lodgement and invasion in distant tissues) the cells were inoculated either subcutaneously or intravenously.

Furthermore, the ability of tumor cells to degrade extracellular matrix produced by smooth muscle cells was used as an in vitro model in which the effects of anticatalytic antibodies against t-PA and u-PA were tested.

MATERIALS AND METHODS

Cell lines

All cell lines were derived from human melanoma metastases. The IF6 (60) and MV3 (61) cell lines were developed from lymph node metastases of two different male patients. The BLM cell line is a subline of BRO (35) and was isolated from lung metastases after subcutaneous inoculation of nude mice with BRO cells. The M14 (31) cell line was obtained from Dr. A. Cochran (John Wayne Clinic, Johnsson Comprehensive Cancer Center, UCLA school of Medicine, Los Angeles, California, USA). The Mel57 (9) cell line was kindly provided by Dr. J. de Vries (the Netherlands Cancer Institute, Amsterdam, the Netherlands). The 530 cell line (59) was established and kindly provided by Dr. P. I. Schrier (University Hospital, Leiden, the Netherlands).

Cell Culture

All cell lines were grown as monolayers in DME medium supplemented with 10% FCS, 2mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. To determine the protein and mRNA levels of plasminogen activators and their inhibitors fresh serum containing medium was added to the cell cultures shortly before they reached confluency, 24 h later when the cells had reached confluency, the conditioned medium was collected and cell extracts were prepared for antigen determination and RNA isolation.

Assay for PA and PAI antigen

Antigen levels of t-PA, u-PA, PAI-1 and PAI-2 were determined in both conditioned media and cell extracts (extracted with 0.5% Triton X-100 and scraped with a rubber policeman) of cultured human melanoma cell lines. The antigen levels in tissue extracts of subcutaneous tumors derived from these cell lines after inoculation in nude mice were also determined. Extracts of subcutaneous tumors in nude mice were prepared using 0.1 M Tris.HCl (pH7.5)/0.1% Tween 80 as a homogenization buffer. The final extracts contained 50 mg tumor tissue/ml.

t-PA antigen was determined using the commercial ELISA Imulyse t-PA (Biopool, Umeå, Sweden). u-PA antigen was determined with a sandwich ELISA, described in detail by Binnema et al. (7). As a standard was used purified t-PA or u-PA which was standardized against the respective International Standard preparations on an activity basis using the specific activities of 500 000 IU/mg t-PA (22) and 100 000 IU/mg u-PA (WHO preparation c66/46) (48).

PAI-1 and PAI-2 antigen were determined using the commercial ELISAs Tintelize Stripwell PAI-1 and Tintelize PAI-2 (Biopool). Detection limits for these assays are approximately 10 ng for t-PA and u-PA, 5 ng for PAI-1 and 2 ng for PAI-2 per ml.

All determinations were performed in duplicate. Serum containing non-conditioned medium was used as control.

mRNA analysis

Cells were washed with PBS at 37°C and lysed in 4 M Guanidium thiocyanate, 25 mM sodium citrate, pH 7.5, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol. RNA was isolated according to Chomczynski and Sacchi (11). The total amount of RNA isolated was determined by measuring the OD_{260} assuming that 1 OD_{260} unit is equivalent to 40 μg RNA.

RNA samples were electrophoresed on a 1.2% denaturating agarose gel containing 7.5% formaldehyde and were transferred to a nylon membrane (Hybond N, Amersham, United Kingdom) using a Vacugene system (Pharmacia, Sweden).

Membranes were hybridized with ^{32}P-labeled cDNA fragments in 7% SDS, 0.5 M $NaHPO_2$, pH 7.2, 10 mM EDTA at 60°C. Blots were routinely washed with 2*SSC, 1% SDS for 1 h at 60°C (1*SSC = 0.15 M NaCl, 0.015 M sodium citrate). cDNA fragments were labeled using the random primer method (Multiprime; Amersham, UK), with ^{32}P-dCTP (specific activity was approximately 10^9 cpm/μg DNA).

Autoradiograms were prepared using Kodak XAR-5 films and intensifying screens at -70°C.

mRNA quantification

mRNA levels were determined as described by Quax et al. (48) using dot blots containing series of dilutions of in vitro transcripts of t-PA, u-PA, PAI-1 and PAI-2 as standards. These RNA transcripts were made using T7 RNA polymerase (Promega, Leiden, the Netherlands). After removal of the DNA templates with RQ1 DNAse (Promega), the amounts of RNA transcripts synthesized were determined by measuring OD_{260}. As an internal standard β-actin was used.

cDNA probes

For the hybridization experiments the following cDNA fragments were used as probes: a 1.9 kb Bgl II fragment of the human t-PA cDNA (62), a 1.0 kb EcoRI-Pst I fragment of the human u-PA cDNA (37), a 1.2 kb PstI fragment of the human PAI-1 cDNA (6), a 1.2 kb EcoRI fragment of the human PAI-2 cDNA, kindly provided by Dr. E.K.O. Kruithof (52) ,and a 1.2 kb PstI fragment of a hamster β-actin cDNA, kindly provided by Dr. W.J. Quax (18).

As a probe for the u-PA receptor mRNA the p-uPAR-1 plasmid containing the complete cDNA for human u-PAR (51) was used after random primer labeling and hybridization performed as described earlier (36).

Extracellular matrix preparation and degradation

Bovine smooth muscle cells (kindly provided by Dr. G. Sperti) were grown to confluency in DME medium supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin in 2 cm^2 culture dishes. After the cultures had reached confluency and the formation of extracellular matrix was started, the cells were incubated for 4 days with medium containing a ^{3}H-amino acid mixture (1 μCi/ml, Amersham). Cells were then lysed using 0.5% Triton X-100 in PBS and the cytoskeleton was removed by 25 mM ammoniumhydroxide treatment. Unincorporated ^{3}H-amino acids were washed from the remaining extracellular matrix using H_2O (twice) and 75% ethanol. Matrices were dried and stored at -20°C until use. Before tumor cells were seeded onto the ^{3}H-labeled matrices, the latter were soaked with medium for 1 hour. For the extracellular matrix degradation assay 10^5 cells/2 cm^2 dish were seeded onto the matrix in 10% FCS containing DME medium or DME medium supplemented with 100 U/ml aprotinin (as plasmin inhibitor) or specific inhibiting antibodies against t-PA (50) or u-PA (7). Antibody concentrations used were approximately 30 μg/ml for anti-t-PA and 300 μg/ml for anti-u-PA. Human plasminogen, purified by affinity chromatography as described previously (63), was added to the medium in all experiments to a final concentration of 0.14 μM. After two days the conditioned medium was removed and the remaining matrix was degraded with 0.25% trypsin (1 hour at 37°C). ^{3}H-amino acid release was determined

and the ^{3}H release by the tumor cells was expressed as percentage of the total amount of ^{3}H released (the sum of the release by the cells and by the trypsin treatment).

u-PA receptor analysis

The presence of u-PAR in the cells was determined essentially according to Nielsen et al. (41). Tumor cells were grown to confluency in DME medium + 10% FCS, detached from the culture dishes using a rubber policeman and washed twice with PBS. After a moderate acid treatment (0.05 M glycine, pH 3.0, 0.1 M NaCl, 3 min, room temperature) to remove the endogenous u-PA from the receptor, cells were lysed in 0.1 M Tris.HCl pH 8.1, 1% Triton X-114, 10 mM EDTA, 100 U/ml aprotinin, 1 mM PMSF and centrifuged for 10 min at 10000x g. The supernatant was stored at -20°C. Diisopropylfluorophosphate (DFP)-treated u-PA (5)(kindly provided by Dr. N. Behrendt) was radiolabeled using Na-^{125}I according the Iodogen procedure (Pierce). u-PA receptor was determined in crude cell extracts by incubation of a sample of the cell extract (of approximately 25000 cells) with ^{125}I labeled DFP treated u-PA, with or without addition of a 100-fold excess of unlabeled u-PA, in 20 μl PBS,0.1% Tween 80 for 1 h at 4°C, followed by incubation with the crosslinking agent disuccinimidyl suberate (2 mM) for 15 min at room temperature and with ammonium acetate (10 mM) for 10 min at room temperature. The samples were then analyzed by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) and autoradiography using Kodak XAR-5 film and intensifying screens at -70°C. Extracts of subcutaneous tumor tissue (50 mg/ml) were prepared by homogenization in 0.2 M Tris.HCl (pH 7.5). Subsequently, membrane fractions were purified by centrifugation of the extracts in an airfuge (Beckman) at

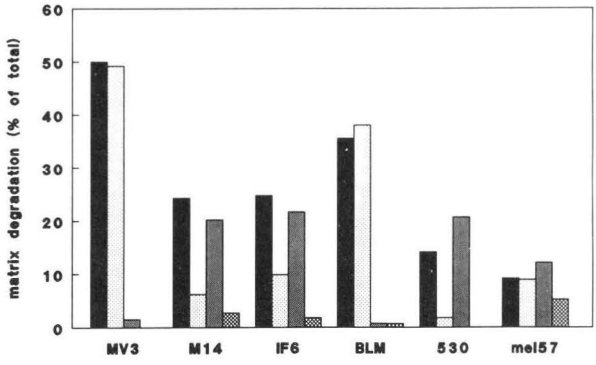


Figure 1. Matrix degradation by human melanoma cell lines.
The indicated melanoma cell lines (10^{5} cells) were incubated for 2 days on radiolabeled extracellular matrix produced by bovine smooth muscle cells, in serum-containing medium supplemented with human plasminogen (0.14 μM), in the presence of 30 μg/ml anti-t-PA IgG (second bar), 300 μg/ml anti-u-PA IgG (third bar) (100 U/ml) aprotinin (fourth bar) and as a control without any additions (first bar). Matrix degradation was measured by the release of radioactivity into the medium. ^{3}H released by tumor cells was expressed as a percentage of the total ^{3}H released by the cells and a subsequent treatment of the matrix with 0.25% trypsin (1 h, 37°C). The data represent the average of three determinations.

130000x g for 15 min. The pellet was resuspended in glycine buffer (0.05 M glycine, pH 3.0, 0.1 M NaCl), centrifuged for 15 min at 130000x g, resuspended in 0.1 M Tris.HCl pH 8.1, 1% Triton X-114, 10 mM EDTA, 100 U/ml aprotinin, 1 mM PMSF, and finally centrifuged for 10 min at 10000x g. Analysis for presence of u-PAR in the supernatant was performed as described above.

Nude mice

Balb/c athymic nude mice (nu/nu) were purchased from the Laboratory Breeding Research Center (Gl. Bomholtgaard, Ry, Denmark) and kept in separate rooms in cages covered with air filters under SPF conditions. Mice were used when 4-6 weeks old and were sex matched.

Tumor cell inoculation

Melanoma cells were harvested from subconfluent cultures by trypsinization, washed twice with serum containing medium, suspended in PBS ($1\text{-}2x10^7$ cells/ml) and inoculated a.) subcutaneously into the lateral thoracic wall to produce tumors at the inoculation site and to produce pulmonary metastases or b.) intravenously into the lateral tail vein to produce lung colonies. $1\text{-}2x10^6$ Tumor cells were injected in both cases. Mice were routinely checked twice a week and sacrificed when signs of illness were noted or, when they remained healthy, 3-4 months after inoculation autopsy was performed and kidney, spleen, liver, lymph nodes and lungs were routinely examined for metastasis both macroscopically and microscopically. Tissue samples from subcutaneous tumors and from the lungs were snap frozen and stored at -70°C for biochemical analysis and immunohistological staining.

Detection of lung metastases

At autopsy lungs were taken for histopathological examination, fixed in formalin, and embedded in paraffin. To avoid missing micrometastases 4 μm haematoxylin and eosin stained sections from at least three different levels were examined for the presence of lung metastases (60,61).

Immunohistochemical staining of u-PA

Indirect immunoperoxidase staining was done with two well characterized polyclonal rabbit antibodies against human u-PA of different origins (7,32) on 4 μm frozen sections of xenograft lesions of all six melanoma cell lines. After aceton fixation sections were processed as described by Nakane and Pierce (40) As second antibody swine-anti-rabbit IgG conjugated to horseradish peroxidase (Dakopatts, Denmark) was used and 3-amino-9-ethylcarbazole as substrate. Harris haematoxylin (Merck, Germany) was used to

counterstain. As a control for specificity of the human u-PA antibodies rabbit non-immune serum or antibodies absorbed with purified human u-PA were used (27).

RESULTS

Extracellular matrix degradation in vitro

The capacity of all 6 melanoma cell lines to degrade extracellular matrix was tested by culturing them on ^{3}H-labeled extracellular matrix produced by bovine smooth muscle cells. Five of the 6 cell lines had the capacity to degrade the matrix (Fig. 1). Only Mel 57 showed no significant degradation of the matrix when compared to the background. In all cell lines studied, except Mel 57, matrix degradation could be inhibited almost completely by aprotinin addition (Fig. 1), indicating the involvement of trypsin-like proteases, probably plasmin, since there was a strong decrease in the matrix degradation when several cell lines were grown in the absence of plasminogen (serum free conditions without addition of plasminogen)(results not shown).

Cultivation in the presence of specific antibodies against t-PA showed a clear decrease in the matrix degradation caused by the M14, IF6 and 530 cells, but had no effect on the matrix degradation by the MV3 and BLM cells. Incubation with antibodies against u-PA showed a strong decrease in MV3- and BLM-mediated matrix degradation, but had no effect on M14-, IF6- and 530-mediated matrix degradation. These results indicate that extracellular matrix degradation is mediated by t-PA in the M14, IF6 and 530 cells and by u-PA in MV3 and BLM cells.

Synthesis of PA and PAI by melanoma cells

To determine which types of plasminogen activators or plasminogen activator inhibitors are produced by the melanoma cell lines tested, preparations of total RNA were analyzed by Northern blot hybridizations, using cDNA probes for t-PA, u-PA, PAI-1 and PAI-2 (Fig. 2). In all the melanoma cell lines studied t-PA mRNA could be detected, while in only two cell lines, MV3 and BLM, u-PA mRNA was detected. PAI-1 and PAI-2 mRNA were also only detectable in these two latter cell lines. mRNA levels and the corresponding protein levels were quantified in cell extracts and in conditioned media in parallel cultures (Table 1). t-PA antigen was detected in all melanoma cell lines studied and more than 96% of the t-PA was found in the culture medium after 24 h incubation. u-PA was only detectable in MV3 and BLM. In this case, however, a significant fraction (16% and 30% respectively) was cell-associated after 24 h incubation. PAI-1 antigen was detected in the MV3 and BLM cell lines only and more than 97% was found in the culture medium. The highest levels of PAI-2 were detected in MV3 and BLM, but also in the other melanoma cell lines small amounts of PAI-2 were detectable

although no mRNA could be detected. In all cases PAI-2 was mainly in a cell-associated form. In general, the mRNA levels corresponded well with the protein levels.

Urokinase cross-linking to cell lysates

Radiolabeled DFP-treated urokinase was incubated and cross-linked with lysates of all 6 melanoma cell lines studied. In all cell lines a 94 kD complex was detected after SDS-PAGE and autoradiography. The instensity of the 94 kD band was similar in all the cell lines except Mel57, which showed considerably less complex formation (figure 3). In all cases complex formation could be prevented almost completely by addition of excess of unlabeled urokinase. These results indicate that ^{125}I-u-PA binding is saturable and that the complexes formed have characteristics similar to u-PA/u-PAR complexes formed observed with other human cell lines (41).

Urokinase receptor mRNA analysis

RNA extracts of all melanoma cell lines were analyzed for the presence of the u-PAR mRNA using Northern blot hybridization with a specific u-PAR cDNA probe (51). In all the cell lines u-PAR mRNA was detectable (fig 4), however the intensity of the hybridization signals varied considerably. The strongest signal was found in RNA extracts from the MV3 and BLM cells.

Tumorigenicity and metastatic behaviour of melanoma cell lines

To assess the tumorigenicity in nude mice, melanoma cells were injected subcutaneously. The data concerning tumor formation at the site of inoculation (tumor take) are

Figure 2. Northern blot analysis of RNA from different human melanoma cell lines.
RNA was isolated as described in Materials and Methods, subjected to electrophoresis and blotted. 10 μg of total RNA was used in each lane. The filters were subsequently hybridized with cDNA fragments of human t-PA, human u-PA, human PAI-1 and human PAI-2. β-actin was used as a control.

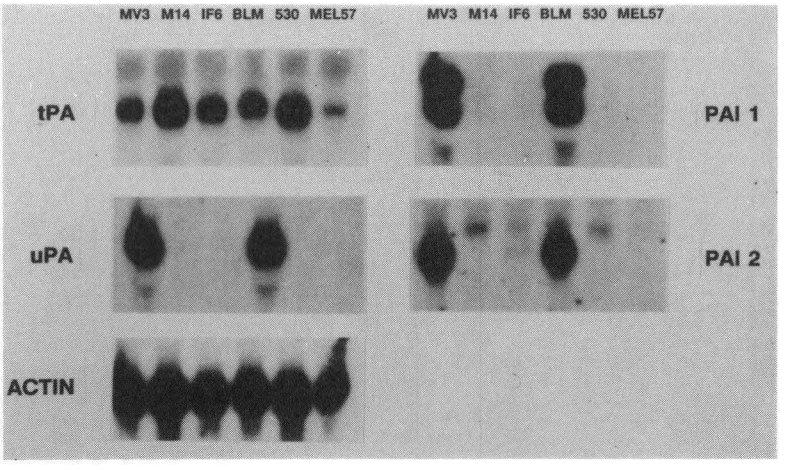
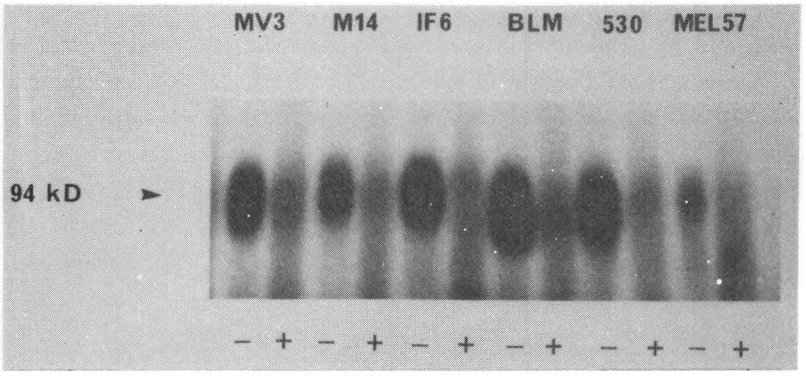


Figure 3. Lysates of human melanoma cell lines were incubated with ^{125}IDFP u-PA in the presence (+) or absence (-) of a 100-fold excess of unlabeled u-PA. As described in Materials and Methods lysates of approximately 2.5x 10^4 cells were used for each lane. After crosslinking with disuccinimidyl suberate, samples were analyzed using SDS-PAGE and visualized using autoradiography.



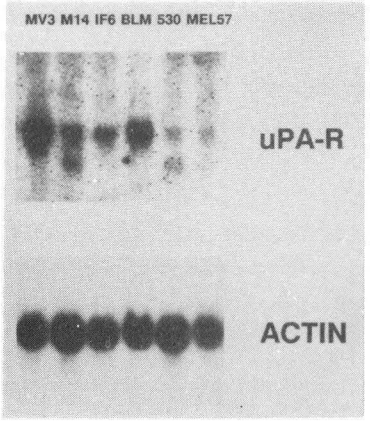


Figure 4. Northern blot analysis of u-PAR mRNA for different human melanoma cell lines. 10 μg of total RNA was used per lane. The filters were hybridized with a cDNA probe for human u-PAR. β-actin was used as a control.

presented in table 2. Subcutaneous tumor development varied from 50% (M14) to 95% (MV3) and tumor latency ranged from 10 days (MV3) to 40 days (530). The capacity to develop lung metastases after subcutaneous inoculation is also presented in table 2. As can be seen, two of the cell lines, IF6 and 530, did not develop metastases at all, two cell lines, M14 and Mel 57, only sporadically, and two cell lines, MV3 and BLM, very frequently. With respect to the non-metastasizing cell lines IF6 and 530, we found in an additional experiment that even 6-7 months after subcutaneous tumor cell inoculation or after inoculation of 10^7 tumor cells in stead of the usual 1-2x10^6 cells, no lung metastases could be observed. The difference in invasive character of BLM and MV3 cells compared with the less- or non-metastasizing other cells lines was also consistently observed in the subcutaneous tumors. As illustrated in figure 5 subcutaneous xenografts of IF6 were encapsulated by host stroma (fig. 5A and 5C), while MV3 (and BLM) cells

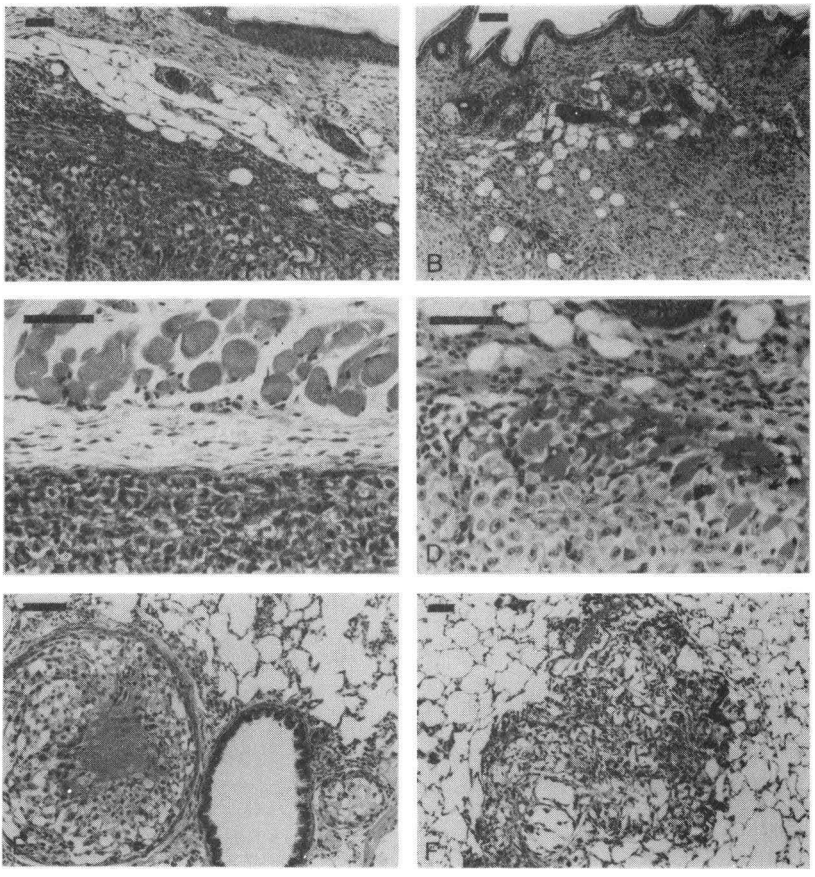


Figure 5. Morphological aspect of subcutaneous tumors after inoculation of IF6 cells (A,C) and MV3 cells (B,D) and of the metastases in lungs of mice subcutaneously inoculated with MV3 cells (E,F). In the lung sections both tumor cell emboli (E) and invasively growing metastases (F) were found.
All sections were stained with haematoxylin and eosin. The indicated bar represents 1 μm.

were not (fig. 5B). In addition, MV3 (and BLM) cells invaded into the subdermal muscles (fig. 5D). As it appears from table 2 the number of lesions found in the lungs of mice that developed metastases varied strongly and both tumor cell emboli (fig. 5E.) and invasively growing lung metastases (fig. 5F.) were observed.

The capacity to develop experimental metastases after intravenous tumor cell inoculation is also presented in table 2. All melanoma cell lines, except IF6, had the capacity to colonize to the lung and form metastases with frequencies between 50% (M14) and 95% (MV3).

Table 1

cell type	t-PA			u-PA		
	mRNA	protein	% protein	mRNA	protein	% protein
	pg	ng	in medium	pg	ng	in medium
MV3	529	342	98%	273	418	84%
M14	409	677	98%	-	-	-
IF6	368	696	96%	-	-	-
BLM	348	209	97%	75	151	70%
530	1064	1286	98%	-	-	-
Mel 57	75	150	99%	-	-	-

cell type	PAI-1			PAI-2		
	mRNA	protein	% protein	mRNA	protein	% protein
	pg	ng	in medium	pg	ng	in medium
MV3	1323	349	98%	1323	308	11%
M14	-	-	-	-	4.8	55%
IF6	-	-	-	-	25	23%
BLM	622	204	97%	498	99	16%
530	-	-	-	-	20	35%
Mel 57	-	-	-	-	6.9	35%

Table 1. PA and PAI mRNA and protein levels of melanoma cell lines in vitro.
Plasminogen activator and plasminogen activator inhibitor protein and mRNA levels per 10^6 cells. mRNA levels in cells extracts were determined by dot blots analysis. Protein levels were determined in cell extracts and in conditioned medium, 24 h after media shift, using ELISA. All determinations were performed at least in duplicate and the average numbers are given. For experimental details see Materials and Methods section. Dashes indicate values below detection limits, i.e. for mRNA: no signal detectable on the corresponding northern blot (figure 2) and for protein: 0.03 ng and 0.13 ng per 10^6 cells for u-PA and PAI-1 respectively.

Detection of plasminogen activator and inhibitor in tumor extracts

Antigen levels in extracts of subcutaneous tumors were determined for t-PA, u-PA and PAI-1 (table 3). Similar to the results obtained with the corresponding cultivated cells (see Table 1), all tumors contained t-PA while u-PA and PAI-1 only were detected in those deriving from the MV3 and BLM cell lines.

Extracts of subcutaneous tumors were also analyzed for the occurrence of u-PA receptor by cross-linking with ^{125}I labeled DFP-treated u-PA. In all tumor extracts studied, except in Mel57, 94 kD complexes could be detected although at a low level (figure 6). After acid treatment of the membrane fraction of the tumor extracts (a procedure which

dissociates endogenous u-PA from its receptor), a strong increase in the complex formation in the MV3 and BLM tumor extracts could be detected (figure 6). This indicates that receptors were present in all the tumors except Mel 57, that the levels of u-PAR in MV3 and BLM tumors were higher than in the tumors of the other cell lines, and that in these two tumors most of the urokinase receptors were occupied by u-PA, which because of a species specificity in the receptor binding (2,21) must be of human origin.

Immunohistochemical localization of u-PA in tumors

To confirm the presence of u-PA in the tumors caused by the melanoma cell lines MV3 and BLM, sections of xenograft lesions were stained immunohistologically using two different u-PA specific polyclonal antibodies. For both antibodies similar results were obtained. Figure 7a and 7c show that BLM cells both in the subcutaneous tumor and in lung metastases derived from these tumors showed a strong, evenly distributed, u-PA staining. For MV3 a similar staining pattern was observed (data not shown). No u-PA staining was detected in the xenografts of any of the other four cell lines studied (figure 7b).

DISCUSSION

It is found that five of the six human melanoma cell lines tested were able to digest in vitro an extracellular matrix synthesized by bovine smooth muscle cells. Degradation of the extracellular matrix was markedly inhibited by antibodies against either t-PA or u-PA. Moreover it was blocked nearly completely by addition of aprotinin to the culture medium, which contained serum and additional plasminogen. These results suggest that plasmin is involved in the matrix degradation. As the extracellular matrices were produced by smooth muscle cells without addition of ascorbic acid to the culture medium they contain little or no collagen (10,29,30). The matrix degradation was mediated virtually only by t-PA in three of the cell lines (M14, IF6 and 530) while in two cell lines (BLM and MV3) it was mediated virtually only by u-PA (figure 1). The finding that u-PA can mediate degradation of extracellular matrix in this system is in agreement with a previous report by Cajot et al. (10) in which mouse L-cells transformed with the human u-PA gene were studied.

All melanoma cell lines studied produced large quantities of t-PA mRNA and antigen, which was mainly secreted into the medium (Table 1). u-PA mRNA and protein, however, were only detected in those cell lines, showing u-PA mediated matrix degradation: BLM and MV3. These were also the only cell lines producing large quantities of PAI-1 mRNA and protein and were also the cells with the highest PAI-2

TABLE 2.

Cell line	subcutaneous inoculation			intravenous inoculation	
	% tumor take[1]	% with lung metastasis[2]	number of metastasis per mouse[3]	% with lung colonies[2]	number of colonies per mouse[3]
MV3	95 (20)	90	9 (1-25)	95 (20)	25 (3-70)
M14	50 (40)	5	25 (2-80)	50 (40)	50 (3-150)
IF6	60 (50)	0	0	0 (25)	0
BLM	75 (60)	50	12 (3-45)	60 (35)	11 (2-30)
530	60 (30)	0	0	80 (15)	300 (10-800)
Mel57	60 (40)	15	14 (3-24)	50 (24)	13 (4-30)

Table 2.
The tumorigenicity and formation of lung metastasis after subcutaneous inoculation of various human melanoma cell lines into nude mice and formation of lung colonies after intravenous injection. 1. Tumor take expressed as the percentage of the mice that gives tumor formation at the subcutaneous inoculation site, number of mice tested is indicated in parenthesis. 2. Frequency of lung lesions after subcutaneous or intravenous inoculation expressed as the percentage of the mice inoculated that developed lesions as detected by microscopic inspection of at least three lung sections from different levels, number of mice tested is indicated in parenthesis. 3. Mean value of number of lesions in each of the mice with metastases or colonies counted in three lung sections from different levels. In parentheses the lowest and the highest number of lesions are shown.

mRNA and protein content, although PAI-2 protein was detected in small amounts in all cell lines. u-PA was in the MV3 and BLM cells partly found in a cell-associated form (Table 1). This portion of urokinase can either be located intracellularly or at the cell surface, specifically bound to the urokinase receptor (31,45,46,55,58). The presence of a specific u-PA receptor in all the melanoma cell cultures was demonstrated by cross-linking experiments with ^{125}I labeled DFP-treated urokinase. Urokinase receptor mRNA was also detectable in all the melanoma cells studied. The u-PAR mRNA analysis showed the strongest signals for MV3 and BLM.

An interesting observation is that although all cell lines produce t-PA, matrix degradation caused by the MV3 and BLM cells could not be inhibited by anti-t-PA while anti-u-PA inhibited the matrix degradation markedly. A possible explanation might be inhibition of t-PA by PAI-1, in contrast u-PA in the medium is probably unaffected by PAI-1 because it is mainly present in its pro-enzyme form (1). Receptor bound u-PA can be inhibited by PAI-1 (13) but the cell surface pathway of u-PA catalyzed plasminogen activation may still be efficient in the degradation of the extracellular matrix because of 1) a different location of u-PA and PAI-1, as it has been observed in some cell types (46); 2) the enhancement of plasmin formation caused by concomitant binding of pro-u-PA and plasminogen to cell surfaces (19,54); 3) a protection of surface bound plasmin from its serum inhibitor α-$_2$ antiplasmin (44,54); and 4) high local concentrations of plasmin at the interphase between the cells and the matrix.

All the human melanoma cell lines could to some extent produce subcutaneous tumors at the site of inoculation. The expression of t-PA, u-PA and PAI-1 in these tumors in vivo corresponded well with the expression in vitro. t-PA was found in extracts of all the tumors, while u-PA and PAI-1 was found only in the MV3 and BLM tumors. The presence of u-PA in the MV3 and BLM tumors and its absence in the other tumors was confirmed by the immunohistochemical studies. In extracts of all the subcutaneous tumors, except the Mel 57 derived tumor, u-PAR was detectable. After mild acid treatment u-PA binding to lysates of the MV3 and BLM tumors increased dramatically, indicating that in these two cases the receptor was already largely occupied by u-PA produced by the human tumor cells, because the human urokinase receptor can not efficiently bind mouse u-PA (2,21).

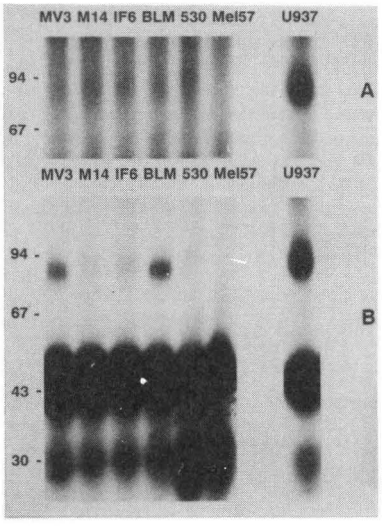


Figure 6. Analysis for u-PA receptor in extracts of subcutaneous tumors derived from human melanoma cell lines (A) or isolated, acid treated, membrane fractions (B) were incubated with ^{125}I DFP u-PA. After crosslinking with disuccinimidylsuberate, samples were analyzed using SDS-PAGE and visualized using autoradiography. Lysates of U937 cells were used as a positive control.

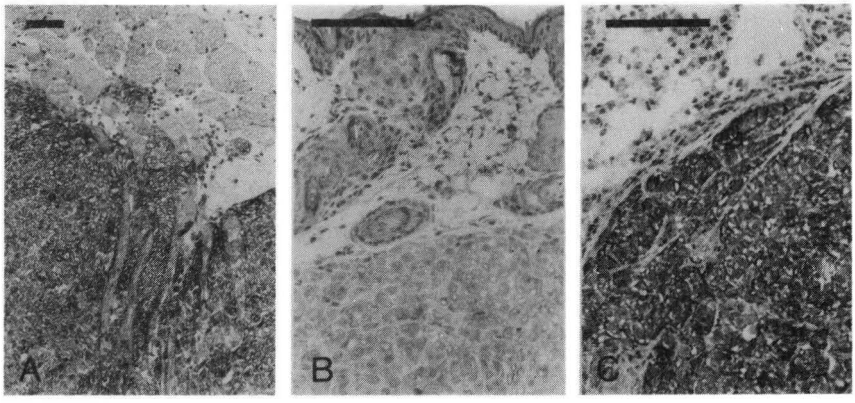


Figure 7. Immunohistochemical staining of tumors for u-PA.
Primary tumors obtained by subcutaneous inoculation of A. BLM cells and B. Mel 57 cells and C. a lung metastasis produced after subcutaneous inoculation of BLM cells. Frozen sections were stained with polyclonal rabbit anti-human-u-PA antibody (6) as described, with nuclear counterstaining. No staining was obtained with the antibody after absorbtion with purified human u-PA (25). Staining with a different polyclonal rabbit anti-human-u-PA antibody (30) gave identical staing. The indicated bar represents 1 μm.

Out of the six cell lines only MV3 and BLM frequently developed lung metastases. These two cell lines were also the only ones having u-PA mediated matrix degradation in vitro and u-PA and PAI-1 production in vitro and in vivo. The melanoma cell lines which only produce t-PA did not, or only sporadically, develop lung metastases after subcutaneous inoculation.
All the human melanoma cell lines, except IF6, caused lung colonies in at least 50% of the mice when inoculated intravenously in the tail vein (table 2). The differences in

TABLE 3.

Tumors from	ng antigen per mg wetweight		
Cell line	t-PA	u-PA	PAI-1
MV3	0.060	0.91	5.3
M14	0.042	-	-
IF6	0.32	-	-
BLM	0.072	0.28	0.56
530	0.29	-	-
Mel57	0.036	-	-

Table 3. PA and PAI protein levels in subcutaneous xenograft lesions.
Protein levels of plasminogen activators and inhibitor type 1 in extracts of subcutaneous tumors derived from human melanoma cell lines in nude mice (dashes indicate levels below detection limits, 0.02 ng/mg and 0.01 ng/mg for u-PA and PAI-1 respectively).

metastasis formation after subcutaneous versus intravenous inoculation suggests that u-PA and/or PAI are involved especially in the early steps in the metastatic cascade and not in the lodgement of melanoma cells in the lung. This conclusion is in contrast to previous reports in which a role of plasminogen activators in formation of lung colonies after intravenous inoculation of tumor cells was suggested in studies with transfection of t-PA and u-PA genes (3) or with inhibition of u-PA by preincubation with anti-catalytic u-PA antibodies (27).

Our findings suggest that the ability to metastasize after subcutaneous inoculation is not only related to u-PA production but possibly also to the production of PAI-1 and PAI-2. In some cell types receptor bound pro-u-PA and u-PA is localized to cell-cell and focal-cell substratum contact sites (28,45,46,54), and it has been proposed that a selective activation of pro-u-PA on some of these contact sites may lead to a directional proteolysis involved in cell migration (45,17). PAI-1 and PAI-2 are effective inhibitors of u-PA even when it its receptor bound (4,13,14,20,47) and it is possible that the inhibitors are required for a finely tuned regulation of the surface plasminogen activation during migration of the cancer cells in the invasive process.

Acknowledgement:
This study was supported in part by the Netherlands Cancer Foundation, The Danish Cancer Society and by the Danish Biotechnology Program, center of Molecular Cell Biology.
Ms. I. Cornelissen and Mr. C. Jansen are acknowledged for their excellent technical support and Dr. H. Verspaget for the fruitful discussions.

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CHAPTER 6

TOWARDS A GENETIC DISSECTION OF THE INVASIVE PHENOTYPE: COMPLEMENTATION OF UROKINASE AND ITS RECEPTOR IN EXTRACELLULAR MATRIX DEGRADATION.

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Sumitted for publication

ABSTRACT

As a first step in a complementation analysis of the invasive process, the respective role of urokinase (u-PA) and its receptor (u-PAR) in extracellular matrix degradation was investigated co-cultivating transfected mouse LB6 cells producing either human u-PA or human u-PAR. While u-PA producing cells alone degrade the extracellular matrix in the presence of plasminogen, u-PAR producing cells do not. Co-cultivation with the receptor-producing cells increases the rate of matrix degradation at least three fold. Immunoprecipitation experiments show that co-cultivation of the two types of cells increases the conversion of the inactive pro-u-PA to active two chain u-PA. The enhancement of matrix degradation and of the pro-u-PA activation requires actual binding of pro-u-PA to the cell membrane since it is inhibited by u-PAR antagonists. Finally, receptor solubilized from the cell surface with phosphatidyl-inositol specific phospholipase C did not activate pro-u-PA in the presence of plasminogen. This type of complementation analysis can now be applied to more complex systems like metastasis in nude mice.

INTRODUCTION

Plasminogen activation is a key process in the regulation of extracellular proteolysis, a process directly related to the migration of cells in both normal and pathological conditions (Danø et al., 1985; Pollanen et al., 1991). The product of plasminogen activation, the broad-spectrum serine protease plasmin, can directly or indirectly degrade protein components of the extracellular matrix and of the basement membrane. The regulation of the conversion of plasminogen to plasmin, mediated by plasminogen activators, is very complex and probably not all components have been identified yet, nor has the precise function of each of the known components been well established. Two distinct plasminogen activators have been described, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA).

Although it is likely that both t-PA and u-PA are involved in cell migration and invasive processes (Axelrod et al, 1989; Cajot et al., 1987; Quax et al., 1990), more knowledge has been accumulated to date for u-PA. u-PA is synthesized and secreted as a single chain pro-enzyme that needs activation to the two-chain active u-PA (Nielsen et al., 1982; Wun et al., 1982; Petersen et al., 1988). The activity of u-PA on the other hand is controlled by specific inhibitors (Sprengers and Kluft, 1987; Andreasen et al., 1990). Finally, u-PA activity can partition between solution and cell surface through the attachment to specific cell-surface receptors (Vassalli et al. 1985; Stoppelli et al. 1985; reviewed in Blasi, 1988). On some cell types receptor-bound u-PA is localized at specific sites on the cell surface,

i.e. the cell to cell and the (focal) cell to substratum contacts (Pollanen et al., 1987, 1988; Hebert and Baker, 1988).

Although the involvement of the plasminogen activator system in cell migration and tissue remodeling processes is well established (Ossowski and Reich, 1983; Ossowski, 1988; Mignatti et al., 1986), the exact mechanism and physiological role of each individual component still needs clarification. In addition, the phenotypic characteristics related to plasminogen activation, i.e. extracellular matrix and basement membrane degradation (Bergman et al., 1986; Cajot et al., 1989), invasion of natural and reconstituted membranes (Mignatti et al., 1986; Reich et al., 1988), metastasis (Ossowski and Reich, 1983; Hearing et al., 1988) and cancer invasion (Skriver et al., 1984; Kristensen et al., 1990; Grøndahl-Hansen et al., 1991) are extremely complex on their own, possibly requiring in addition to plasminogen activation system other proteolytic activities like collagenase, which may in turn be controlled by inhibitors, and presumably receptors (Trygvason et al., 1987).

A deeper understanding of the role of some of the components of the plasminogen activation system in the various biological functions in which it participates can be obtained through a genetic dissection of the system. To this gaol we have set up a reverse in vitro complementation system as an initial step in this direction. Cells unable to carry out plasminogen activation can be modified by transfection with individual genes of the PA system. The resulting cells would not be able to carry out the whole reaction, but should be able to complement each other.

The mouse LB6 cells (Corsaro et al., 1981) represent a suitable host cell line for this purpose as they produce little or no u-PA and t-PA (this paper) nor interstitial collagenase (Ossowski, personal communication). In addition, LB6 cells can be considered to lack binding capacity for human u-PA, since binding of u-PA to its receptor is largely species-specific (Vassalli and Belin, personal communication; Appella et al., 1987; Estreicher et al., 1989). Finally, as demonstrated here, they have a very poor capacity to degrade the extracellular matrix. We have previously cloned the genes for several components of the human u-PA system: u-PA (Riccio et al., 1985), the inhibitor PAI-1 (Andreasen et al., 1986; Bosma et al., 1987) and the u-PA receptor (Roldan et al., 1990). We now have constructed mouse LB6 cells that express either human u-PA or the human u-PA receptor, and have tested their

respective role in degrading the extracellular matrix. We here report complementation between these cell lines, carrying either the gene for u-PA or for u-PA receptor, and demonstrate a role of receptor in the degradation of the extracellular matrix via the activation of the zymogen pro-u-PA.

Characterization of u-PA and u-PA receptor production by transfected LB6 clones

The synthesis of the human pro-u-PA gene product in LB6 clone F cells, but not by parental LB6 nor LB6 clone 19 cells, was demonstrated by zymography (Fig. 1a) and was further verified by immunoprecipitation and measurement of enzymatic activity with synthetic S2444 substrate (not shown). The presence of the human u-PA receptor in LB6 clone 19 cells, and not in LB6 nor LB6 clone F cells, was verified using binding cross-linking assays with iodinated human u-PA (Fig. 1b) or its amino terminal fragment (ATF).

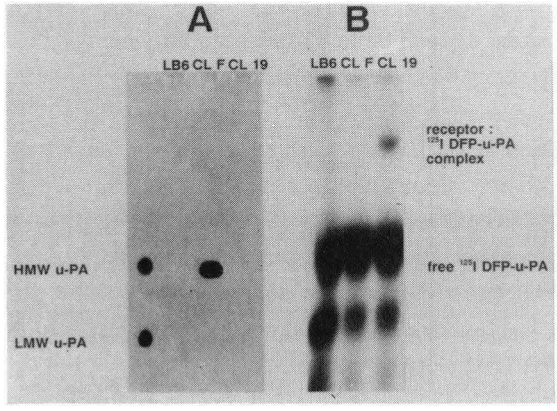

Figure 1: Charaterization of the u-PA and u-PAR production by mouse LB6 cells and its derivatives.
A.: SDS-PAGE and fibrin zymography of conditioned medium of control LB6 cells, u-PA producing LB6 clone F cells (Cl F) and u-PAR producing LB6 clone 19 cells (Cl 19). The leftmost lane shows the zymography of a preparation of human u-PA (British Standard) with activity at both the high molecular weight (HMW) and the low molecular weight (LMW) u-PA bands.
B: SDS-PAGE analysis of extracts of the three cell lines (see part A) after binding and cross-linking to ^{125}I-DFP-treatedu-PA.

Extracellular matrix degradation by u-PA producing cells

All cell lines were tested for their capacity to degrade extracellular matrix in vitro in serum-containing medium with additional human plasminogen. In Figure 2 the matrix degradation after 24 hours is shown for pro-u-PA producing cells (LB6 clone F), u-PAR producing cells (LB6 clone 19), and control cells (LB6). LB6 clone F cells, producing u-PA, were able to degrade the matrix. Addition of polyclonal anti-human u-PA IgGs strongly inhibited the degradation process. Trasylol, a potent inhibitor of trypsin-like proteases including plasmin, completely inhibited the degradation. These results point to an involvement of urokinase and plasminogen activation in the matrix degradation process. Untransfected LB6 cells or cells expressing the human u-PA receptor (LB6 clone

19) were not able to degrade the extracellular matrix. These data show that the inability of mouse LB6 cells to degrade extracellular matrix can be circumvented by transfection of the human u-PA gene indicating that u-PA is the only component required for extracellular matrix degradation missing in the LB6 cells.

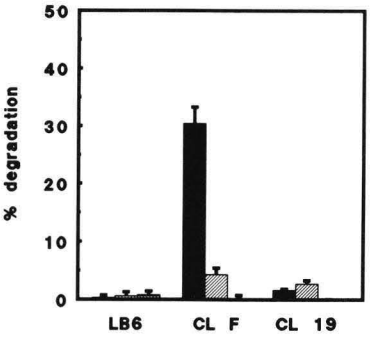

Figure 2: Effect of plasmin and u-PA inhibitors on extracellular matrix degradation by Mouse LB6 cells and derivatives. The capacity of LB6 cells, u-PA producing LB6 Clone F cells (Cl F) and u-PAR producing LB6 Clone 19 (Cl 19) cells to degrade ^{3}H labeled bovine smooth muscle cell extracellular matrix was determined of a 10^{5} cells per experiment. Cells were cultured in 10% fetal calf serum containing DME medium supplemented with 0.14μM human plasminogen. No addition : filled bars; anti-human u-PA IgG (30 μg/ml); hatched bar; Trasylol (100 U/ml): dotted bars. Matrix degradation was determined after 24 hours incubation as described under materials and methods. The data represent the average of three experiments with standard deviation.

Role of u-PA receptor in extracellular matrix degradation

The role of the cell surface u-PA receptor in extracellular matrix degradation was studied using co-culturing experiments. Mouse LB6 clone F, LB6 clone 19 expressing human u-PA or human u-PAR respectively, and control LB6 cells were co-cultured in various combinations and ratios on ^{3}H-labeled extracellular matrices. Subsequently, the rate of matrix solubilization was determined. Co-cultures contained 5%, 7.5% or 10% LB6 clone F in combination with either LB6 clone 19 or control LB6 cells. Untransfected mouse LB6 cells in combination with u-PAR expressing cells (LB6 clone 19) were used as a negative control and had a negligible matrix degrading activity which was subtracted (Fig. 3). Matrix degradation in co-cultures of control LB6 with LB6 clone F cells was found to be dependent on the incubation time and on the number of LB6 clone F cells (Fig. 3). In all cases, matrix degradation by LB6 clone F cells was clearly enhanced by the co-cultivation with receptor-producing LB6
clone 19 cells (Fig. 3). While the latter cells have hardly any capacity to degrade extracellular matrix (see also Fig. 2) they are able to stimulate matrix degradation

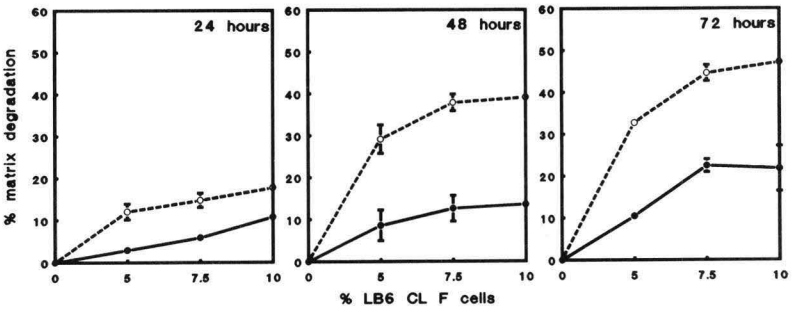

Figure 3: Stimulation of extracellular matrix degradation of u-PA producing LB6 clone F by co-cultivation with u-PAR producing LB6 clone 19 cells. Different percentage of LB6 clone F cells were cocultured with either control LB6 or u-PAR producing LB6 clone 19 cells (the total number of cells being in each case 10^5). Matrix degradation was determined after 24, 48 and 72 hours of incubation as described under materials and methods. The data represent the average of three experiments with standard deviation.
Co-cultures of clone F and control LB6 cells: ●---● , Co-culture of clone F and LB6 clone 19 cells: o---o.

considerably when co-cultivated with LB6 clone F cells. Also in this case matrix degradation is totally blocked by Trasylol and anti-u-PA antibodies. These results suggest that at low pro-u-PA concentrations, the presence of the receptor increases the ability of u-PA to degrade the matrix proteins.

The specificity of the effect of u-PA receptor-producing cells has been tested with a synthetic peptide representing the binding sequence of human u-PA, u-PA[12-32(ala19)] which is able to compete with u-PA for binding to the receptor (Appella et al., 1987). As shown in Fig. 4, this peptide nearly completely inhibited the enhancement of matrix degradation in the co-cultivation of 7.5% clone F cells with clone 19 cells. On the contrary, the synthetic peptide u-PA[13-33(ala20)], representing the binding sequence of the mouse enzyme, which does not bind to the human receptor (Appella et al., 1987; Estreicher et al., 1989), had little or no effect.

The synthetic peptides, while specific, have a relatively low affinity for the receptor (Appella et al., 1987) and possibly may have a short half life in culture conditions. Therefore we have performed competition studies with a novel recombinant u-PA derivative, ΔF-u-PA, which contains the 164 amino terminal amino acids of u-PA and a 30 amino acids carboxy-terminal u-PA-unrelated extension. ΔF-u-PA contains the receptor binding domain of u-PA, but lacks the protease domain; it thus cannot convert plasminogen to active plasmin but competes for binding to the receptor (N. Pedersen & F. Blasi, unpublished). As a source of ΔF-u-PA, conditioned medium of LB6 cells stably

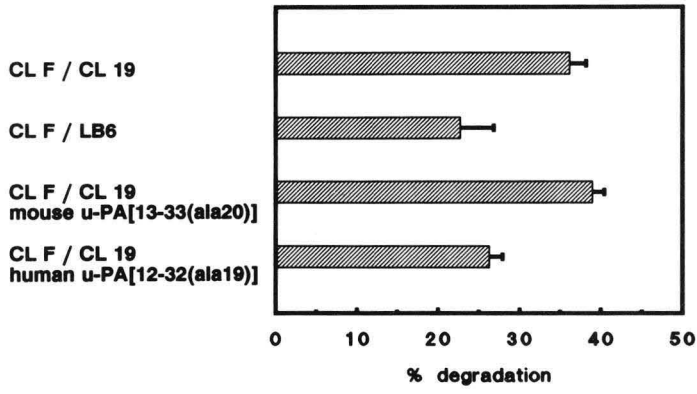

Figure 4: Inhibition of matrix degradation by u-PAR binding peptides. Extracellular matrix degradation by co-cultures of u-PA producing LB6 clone F cells (7.5%) with u-PAR producing LB6 clone 19 cells was determined in the presence and asence of mouse and human u-PA receptor binding peptides. 10^5 cells were seeded on the matrix, 7.5% clone F/ 92.5% LB6 (Cl F/LB6) or 7.5% clone F/ 92.5% clone 19 (Cl F/Cl 19). The latter were also incubated in the presence of 10 μM mouse u-PA [13-33(ala20)] or 10 μM human u-PA [12-32(ala19)] peptides. Matrix degradation was determined after 72 h as described in materials and methods. The data represent the average of three experiments with standard deviation.

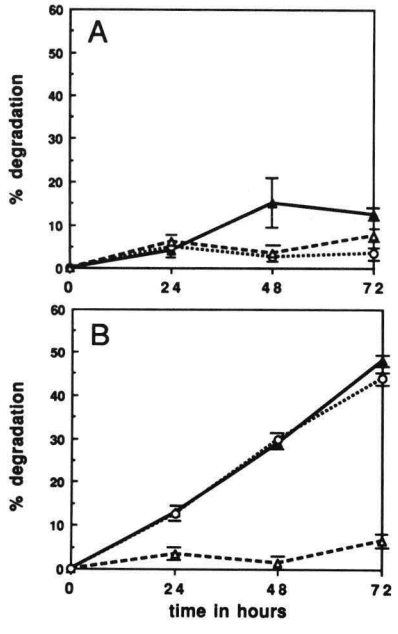

Figure 5: Inhibition of matrix degradation by a receptor-binding u-PA antagonist. Matrix degradation was assessed at different times on co-cultures of LB6 clone F (7.5%) and LB6 control cells (92.5%) (panel A), or clone F cells (7.5%) and LB6 clone 19 (92.5%) (panel B) [total 10^5 cells]. The experiments were carried out in the presence (▵--▵) of the u-PA mutant ATF u-PA or of the medium of control LB6/RSVneo cells (O--O) or without any addition (▲--▲). As a source of ATF-u-PA the conditioned medium of LB6 clone ΔF cells (10% of the total medium) was used. The data represent the average of three experiments with standard deviation.

transfected with the variant u-PA gene and secreting the truncated recombinant ΔF-u-PA,was used. As shown in Figure 5b., the presence of 10% conditioned medium of LB6 clone ΔF-u-PA strongly
inhibits (about 90%) the enhancement of matrix degradation by co-cultures of u-PA producing LB6 clone F cells and receptor producing LB6 clone 19 cells (in a 7.5% to 92.5% ratio) while conditioned medium of control cells (i.e. LB6 cells transfected with only a RSV-neo plasmid) had very little effect. ΔF-u-PA conditioned medium, containing ATF, decreases the degradation of extracellular matrix to a level even lower than that attained by the combination LB6/LB6-clone F cells, i.e. in the absence of receptor (fig. 5b.). The ΔF-u-PA conditioned medium, however, is also able to inhibit the degradation attained by the combination of u-PA producing LB6-clone F and control LB6 cells (7.5% to 92.5% ratio), by the same degree as control medium (fig. 5a.), i.e. to less than 50%. The results are therefore in line with the possibility that the enhancement of extracellular matrix degradation is directly correlated with the binding of the product of the LB6-clone F cells to the receptor in LB6-clone 19 cells.

The presence of the u-PA receptor increases the conversion of single chain u-PA to two chain u-PA.

The product of the u-PA gene is the single-chain pro-enzyme pro-u-PA (Petersen et al., 1988). *In vitro* conversion of the soluble or receptor-bound pro-u-PA to the two-chain form by plasmin results in the formation of the fully active enzyme (Cubellis et al., 1986). Kinetic data show that concomitant binding of u-PA (to its receptor) and plasminogen to cell surfaces increases plasmin generation primarily by increasing the rate of pro-u-PA activation (Ellis et al., 1989). LB6 clone F cells produce pro-u-PA, and degrade the extracellular matrix in an u-PA-dependent way: therefore pro-u-PA must have been converted to the active two-chain u-PA. This may well occur since the cells were cultured in serum that contains plasminogen and plasminogen was added to the system, so that plasmin could be present as a contaminant or as a product of the low intrinsic pro-u-PA activity. In co-cultivation with LB6 clone 19 cells, a stimulation of the extracellular matrix degrading activity is observed. Thus the presence of receptor and possibly binding to receptor may have increased the rate of conversion of single chain to two chain u-PA. To clarify this issue we have co-cultivated u-PA producing LB6 clone F cells (10%) with untransformed LB6 or LB6 clone 19 cells for 12 hours in serum containing medium, labeled the cells with ^{35}S, and determined the levels of single vs. two chain u-PA in the medium and in the cell-surface bound form by immunoprecipitation with 5B4 monoclonal antiboby and SDS-PAGE analysis. As shown in figure 6 the conditioned medium of co-cultured clone F/control LB6 cells contains mainly single chain pro-u-PA and only limited amounts of two chain u-PA. The higher molecular weight band may represent a complex of active u-PA with inhibitors or more likely a contaminant in the

immunoprecipitation. On the other hand, in the medium from co-cultured clone F/clone 19 cells, about 50% of the u-PA was in the two chain form.

In order to test the state of receptor bound pro-u-PA, cells were acid washed and the acid wash immunoprecipitated. As expected, the surface of co-cultured clone F/control LB6 cells had no cell-attached u-PA, whereas both single chain and, predominantly, two chain u-PA can be dissociated from co-cultured clone F/clone 19 cells. This result can be reproduced by addition of limiting amounts of ^{35}S-labeled pro-u-PA (in the form of ^{35}S-labeled LB6 clone F conditioned medium) to LB6 clone 19 or LB6 control cells and determination of the ratio of single to two chain u-PA in the medium and on the cell surface (Fig. 7). In the presence of LB6 clone 19 cells, labeled u-PA was no longer detectable by the immunoprecipitation technique in the medium after 3.5 hours incubation, rather it could be found receptor-bound; under these conditions, it is mostly in the two-chain form. In contrast, most of the u-PA in the medium of LB6 cells was still in the single chain form.

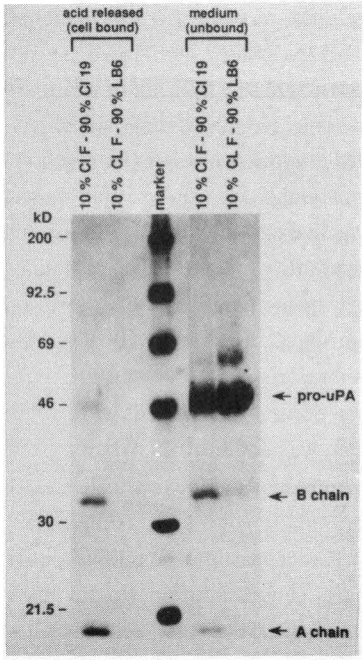

Figure 6: Receptor-bound pro-u-PA is preferentially converted to two-chain (active) u-PA. Complementation of pro-u-PA and u-PAR producing cells. 10% LB6 Cl F cells and 90% LB6 Cl 19 or LB6 cells were plated 12 hours prior to labeling with ^{35}S-methionine and ^{35}S-cysteine for 9 hrs.
The media (unbound) and acid released (cell bound) material was immunoprecipitated with the anti-human uPA monoclonal antibody 5B4 and analyzed by SDS-PAGE under reducing conditions and fluorography.

We have further tested whether, in the presence of plasminogen, it is the binding to the receptor per se that induces the conversion of single chain to two chain u-PA by analyzing the fate of pro-u-PA when it is in the presence of a soluble form of the receptor rather than on the cell surface. It has recently been established that the u-PA receptor is attached to the cell surface via a glycolipid anchor and can be solubilized by treatment with Bacillus cereus phophatidyl-inositol specific phospholipase-C (PI-PLC)

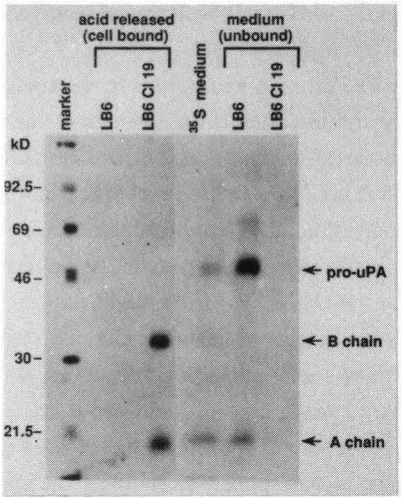

Figure 7: Preferential conversion of exogenous receptor-bound pro-u-PA to the two-chain (active) form. 200 µl ^{35}S-labelled LB6 C! F medium and 2.8 ml fresh medium was added to a semiconfluent 25 cm^2 flask of either LB6 Cl 19 or LB6 cells. After incubation for 3.5 hrs, the medium (unbound) and acid released (cell bound) u-PA was immunoprecipitated with the anti-human u-PA monoclonal antibody 5B4 and analyzed by SDS-PAGE under reducing conditions and fluorography.

(Ploug et al., 1991). Therefore, we prepared soluble u-PA receptor from LB6 clone 19 cells by treating them with PI-PLC. The presence and the binding-activity of the u-PA receptor in the supernatant of the PI-PLC treated cells was tested by cross-linking to ^{125}I-ATF (fig. 8b). Medium containing soluble receptor was incubated with labeled ^{35}S-pro-u-PA (conditioned medium of LB6 clone F cells) for 18 hrs in the presence or absence of exogenous plasmin (100 ng/ml). The reaction mixtures were analyzed by immunoprecipitation and SDS-PAGE (fig. 8a). The presence of a solubilized u-PA receptor did not change the ratio of single vs. two chain u-PA, although plasminogen is present in the serum containing medium. On the other hand, exogenous plasmin quantitatively converted the single chain in the two chain form, independent of the presence of the solubilized receptor.

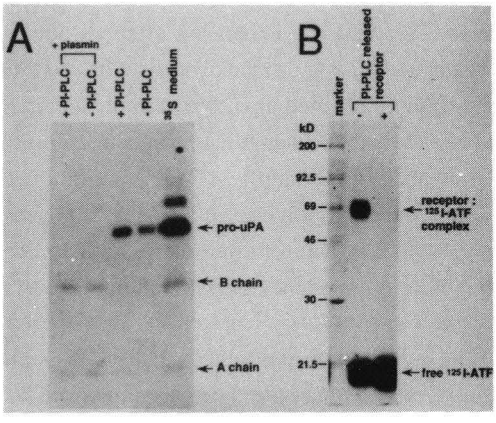

Figure 8: Binding to a solubilized form of u-PA receptor is not sufficient for preferential conversion of pro-u-PA to two chain (active) u-PA. Solubilized u-PA receptor was prepared by incubating a confluent 75 cm^2 flask of LB6 CL 19 cells with 500 µl DMEM containing 1 U/ml PI-PLC.
PANEL A: 200 µl of the solubilized u-PA receptor or 200 µl DMEM was incubated with 100 µl of medium containing ^{35}S labelled pro-u-PA for 18 hrs with or without addition of 100 ng/ml plasmin. The u-PA was immunoprecipitated with the anti-human u-PA monoclonal antibody 5B4 and analysed by PAGE and fluorography. PANEL B: cross-linking of the medium of PI-PLC treated cells with ^{125}I-ATF with (+) and without (-) unlabeled 100nM ATF as competitor. Molecular weight markers are not shown.

DISCUSSION

In the model system presented in this paper the role of u-PA and u-PAR in extracellular matrix degradation could be studied separately and in combination. We show that the produced components are biologically active, that u-PA mediates proteolytic degradation of extracellular matrix, and that u-PAR binds u-PA produced by other cells. Furthermore an enhancement of the effect of u-PA on matrix degradation was demonstrated, due to binding of u-PA to the receptor. It was shown that, unlike the parental LB6 cells, only the u-PA producing cells were able to degrade the ^{3}H-labeled extracellular matrix; u-PAR producing LB6 cells had no or only a very low capacity to degrade the matrix. The key role for u-PA was indicated by the strong inhibition of matrix degradation by anti-u-PA antibodies (fig. 2). The complete inhibition by Trasylol further suggested that matrix degradation is plasmin dependent, a phenomenon also observed by (Cajot et al., 1989). The extracellular matrix produced by smooth muscle cells appears to be a suitable model system to study the plasmin since smooth muscle cells are thought to produce under normal cell culturing conditions collagen-poor matrices, unless ascorbic acid is added to the culture medium (Jones et al., 1980). When matrix degradation was assayed using co-cultures of u-PA and u-PAR producing cells, an enhancement of the extracellular matrix degradation was observed (Fig.3). This enhancement effect is due to the binding of u-PA, produced by the clone F cells, to the u-PAR of the clone 19 cells. This is shown by the specific inhibition of the enhancement by the antagonist synthetic peptide human u-PA[12-32(ala19)] (Fig. 4), while peptide mouse u-PA[13-33(ala20)] had no effect, as expected from the species specificity of the binding. Competition experiments were also performed using ΔF-u-PA (i.e. a recombinant amino terminal fragment of u-PA) as a competitor. This recombinant ATF, ΔF-u-PA, behaves in exactly the same way as the natural ATF (N. Pedersen & F. Blasi, unpublished), in having a much higher affinity for the receptor than the synthetic peptides. ΔF-u-PA totally inhibits the enhancement of the matrix degradation (Fig. 5), again demonstrating that the enhancing effect is due to binding of u-PA to its receptor. If co-cultures of u-PA producing cells with u-PAR producing cells are incubated with ΔF-u-PA conditioned medium (10% of total medium), matrix degradation is decreased to even lower levels than when u-PA producing cells are incubated with control cells. Control experiments showed that the ΔF-u-PA conditioned medium has a low inhibitory effect towards matrix degration caused by u-PA producing cells alone (i.e. in the absence of the receptor). In fact, control experiments showed that in vitro plasminogen activation by LB6 clone F conditioned medium was inhibited by ΔF-u-PA conditioned medium.

Although secreted pro-u-PA alone mediates matrix degradation, its activity is strongly enhanced in the presence of u-PAR. It has been shown that plasmin generation by pro-u-PA is strongly enhanced when the activity is measured in the presence of human U937

cells, due to the binding of pro-u-PA to the surface receptors. Kinetic data show that the stimulating effect of the receptor is not due to an increase in the activity of u-PA, but rather to a faster rate of activation of single chain pro-u-PA (Ellis et al., 1989). Since clone F cells express u-PA in a proenzyme form, the enhancement of extracellular matrix degradation by the u-PAR producing cells is likely to be due to the acceleration of the pro-u-PA activation. And indeed under the experimental conditions used, the presence of u-PA-receptor producing cells results in an increased ratio of two-chain vs. single chain u-PA in the medium; also, most of the added pro-u-PA is found attached to the receptor largely in the activated form (figs. 6 and 7). Finally, we have shown (Fig. 8) that receptor binding per se is not sufficient to activate pro-u-PA; in the presence of a solubilized form of the receptor, no pro-u-PA activation was observed. Possibly an intact cell surface integrated system is required because it provides not only the u-PAR but also the plasminogen receptor.

Moreover binding of both components to the cell surface might cause the appropriate steric relationship (proximity) for pro-u-PA activation. After addition of plasmin, pro-u-PA is activated both in the presence and absence of solubilized receptor. It is therefore possible that a crucial factor introduced by the cell surface binding is a mechanism for initiation of the plasminogen activation system.

The data provided agree and give physiological significance to the previous findings indicating an important role for the u-PA receptor in cellular invasiveness (Ossowski, 1988) and showing that surface bound pro-u-PA can catalyze plasmin formation from the surface-bound plasminogen (Ellis et al., 1989; Stephens et al., 1989). The apparent contradiction with the findings that expression of human pro-u-PA in ras-transformed NIH-3T3 mouse cells that cannot bind the human u-PA (Estreicher et al., 1989), caused an increase in the lung colonizing ability (Axelrod et al., 1989) and that transformation of mouse L-cells with human u-PA could induce extracellular matrix degradation and invasive growth of these cells (Cajot et al., 1989), can also be explained by our data. In our experiments the enhancement effect of receptor binding of u-PA was most prominent at low u-PA concentrations. If the transformed cells in studies mentioned above produce high enough pro-u-PA concentrations, enough active u-PA is generated to allow matrix degradation. At lower concentrations the presence of receptor is required to attain a sufficiently high local activity which allows efficient proteolysis to occur.

Many cultured tumor cells have their surface receptors saturated with endogenous u-PA and this has been suggested to be an important feature in the establishment of the invasive phenotype (Stoppelli et al., 1986; see Blasi, 1988, for further references). The results reported in this paper, while identifying a physiological and biochemical basis for such property, also show that not only endogenous u-PA but also u-PA produced by other cells can bind the u-PA receptor with the same results. This phenomenon may not only occur under experimental conditions. Recent immunohistochemical and in situ

hybridization studies have shown that u-PA and its receptor can be produced by different cell populations in human colon adenocarcinomas, u-PA being produced by fibroblast-like cells in the tumor stroma, and u-PAR by cancer cells located at the invasive foci, indicating a cooperativity between the stromal and the cancer cells in generating cell surface proteolysis (Grøndahl-Hansen et al., 1991; C.Pyke, personal communication).

MATERIALS AND METHODS

Materials

Affigel-bound monoclonal antibody 5B4 (Nolli et al., 1986) was a generous gift of Dr. M.L. Nolli (Lepetit SpA, Italy). Anticatalytic polyclonal antibodies against u-PA have been previously described and characterized (Binnema et al., 1986). Synthetic peptides human u-PA[12-32(ala19)] and mouse u-PA[13-33(ala20)] have been previously described (Appella et al., 1987). The human peptide was dissolved in DMSO and then diluted 100 fold in medium. The mouse peptide was dissolved directly in water and dilluted in medium. Phosphatidylinositol-specific phospholipase C from Bacillus cereus was obtained from Boehringer Mannheim.

Cell culture

Mouse LB6 cells transfected with the human u-PA receptor cDNA under the control of the SV40 promoter and expressing the surface u-PA receptor (LB6 clone 19) have been described previously (Roldan et al., 1990). LB6 cells expressing the human u-PA gene (LB6 clone F) have been obtained following the same procedure as described before (Nolli et al., 1989). LB6 cells - ΔF-u-PA (Pedersen and Blasi, unpublished) were used to produce a conditioned medium rich in ΔF-u-PA (i.e. a recombinant aminoterminal fragment (ATF) of u-PA) which was withdrawn from confluent cultures. Untransfected mouse LB6 cells or LB6 cells transfected with the RSV-neo gene (Gorman et al., 1982) have been used as control. Cells were routinely cultured in DME medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Between the experiments the cells were propagated in medium containing 0.4 mg/ml G418 in order to keep the established cell lines under mild selective pressure.

Fibrin autography

Fibrin autography was performed essentially as described by (Granelli-Piperno and Reich, 1978). u-PA (British standard urokinase) was used as a standard since it contains both the high and low molecular weight forms of u-PA.

Immunoprecipitation of u-PA and pro-u-PA

Production of u-PA by cultured cells was measured by a previously published ELISA (Binnema et al., 1986).

For biosynthetic labeling, 4 x 10^6 cells were plated for 12 hours in a T75 flask, starved for 45 minutes in serum-free, methionine- and cysteine-free medium, and labeled with ^{35}S-L-methionine and 35-S-L cysteine (120 μCi/ml) in methionine- and cysteine-free medium supplemented with 5% FCS for 9 hours. For immunoprecipitation, medium was collected and Trasylol and PMSF added (to 1% and 2 mM, respectively), supplemented with 1/3 of the volume of 0.2 M potassium phosphate buffer pH 7.0 containing 2.0 M NaCl and 0.4% Triton X-100, cleared by centrifugation and stored at -20°C. Immunoprecipitation was carried out on media using anti-u-PA monoclonal antibody 5B4, directed towards the kringle domain of the A chain (Nolli et al., 1986), as previously described (Stoppelli et al., 1986). Immunoprecipitates were analysed by SDS polyacrylamide gel electrophoresis (13%) under reducing conditions. In co-culture experiments the cells were labeled as described above, but for 9 hours, and medium or acid wash (see below) collected and immunoprecipitated. In this case 3 ml of labeled medium was added to 35 μl 5B4-agarose beads and incubated 1 hr at room temperature, then overnight at 4 °C by gentle rocking. After washing with the potassium phosphate-NaCl-triton buffer, the agarose beads were eluted with 0.1 M acetic acid pH3 in 1 M NaCl.

To dissociate any ligand from the cell surface the cells were washed 3 times with PBS, treated for 5 minutes at room temperature with 50 mM glycine pH 3.0 in 0.1 M NaCl, then neutralized with 0.5 M Hepes in 0.1 M NaCl (Stoppelli et al., 1986). The acid washes (final volume 3 ml) were cleared by centrifugation and supplemented with Trasylol, PMSF and potassium phosphate-NaCl-Triton X-100 buffer and immunoprecipitated as described above.

In the experiments in which the state of receptor-bound exogenous pro-u-PA was analyzed, 200 μl of ^{35}S-labeled LB6-clone F medium and 2.8 ml unconditioned medium were added to semiconfluent 25 cm^{25} flask containing either LB6 of LB6-clone 19 cells, and incubated for 3.5 hrs. at 37°C in 10% CO_2 atmosphere. The whole 3 ml were immunoprecipitated as described above.

Phosphatidylinositol-specific Phospholipase C (PI-PLC) release of u-PA receptor from LB6 clone 19 cells

Approximately 6 x 10^6 cells were washed in DME medium containing 25 mM Hepes and incubated at 37°C for 1 hr in 0.5 ml of DMEM-Hepes containing 10 μl PI-PLC from Bacillus cereus (final concentration 0.4 μg/ml). At the end of the incubation, the medium was collected, cleared by centrifugation and stored at -20°C. Conditioned medium (1 ml) of LB6-clone 19 cells was added to 200 μl of fresh medium or of supernatant of the PI-

PLC treatment and incubated with 200 μl of labeled LB6-clone F cells, with or without plasmin (100 ng/ml) at room temperature for 18 hrs. At the end of the incubation the whole mixture was immunoprecipitated as described above.

Cross-linking of iodinated u-PA or ATF to the u-PA receptor

Diisopropylfluorophosphate (DFP) treated human u-PA or the amino terminal fragment of u-PA, ATF, (Stoppelli et al., 1985) were iodinated and cross-linked the u-PA receptor as previously described (Behrendt et al., 1990; Ploug et al., 1991).

Extracellular matrix preparation and degradation

Extracellular matrices were produced in vitro by bovine smooth muscle cells. Bovine smooth muscle cells (kindly provided by Dr. G. Sperti) were grown to confluence in DME medium supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin in 2 cm^2 culture dishes. After cells had reached confluency and formation of extracellular matrix was started, the cells were incubated for 4 days with DME medium containing a ^{3}H-amino acid mixture (1 μCi/ml, Amersham). Cells were then lysed using 0.5% Triton X-100 in PBS and the cytoskeleton was removed by 25 mM ammoniumhydroxide treatment. Unincorporated ^{3}H-amino acids were washed from the remaining extracellular matrix using two water washes and 75% ethanol. Matrices were dried and stored at -20°C until use. To assay for matrix degradation activity, cells (10^5/dish) were seeded onto ^{3}H-labeled matrices, presoaked with medium for 1 hour. Cells were seeded onto the matrix in 10% FCS containig DME medium or in DME medium supplemented with specific inhibitors (as 100 U/ml Trasylol or inhibiting antibodies against u-PA (30 μg/ml), or conditioned medium from LB6 or LB6/ΔF u-PA cells). In all experiments plasminogen was added to a final concentration of 0.14μM. After 24, 48 and 72 hours of incubation, the conditioned medium was removed and the remaining matrix was degraded with 0.25% trypsin (1 hour at 37°C). ^{3}H-amino acid released by the mouse LB6 cells was determined and expressed as percentage of the total amount of ^{3}H released by the cells plus that solubilized by trypsin treatment.

Acknowledgements:

The authors are indebted to Drs. Ettore Appella, Alex van der Eb and Hein Verspaget for the many fruitful discussions, to Eva Gertmann for her invaluable help and to the following colleagues provided important reagents: Dr. M.L. Nolli the 5B4 monoclonal antibody, Dr. Jack Henkin the ATF, Dr. Giovanni Sperti the Bovine Smooth Muscle cells.

The work has been supported by grants of the Danish Cancer Society and the Danish Biotechnology Program, Center for Molecular Cell Biology. MTM was a fellow of the Italian Association for Cancer Research (AIRC).

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CHAPTER 7

CELL SURFACE PLASMINOGEN ACTIVATION IS INVOLVED IN FUSION OF HUMAN MYOGENENIC CELLS IN VITRO.

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ABSTRACT

Differentiation of myogenic stem cells can be mimicked in vitro using primary cultures of myogenic stem cells. During in vitro muscle cell differentiation the expression of the various components of the plasminogen activation system is coordinately regulated. mRNA levels and activities of tissue-type and urokinase-type plasminogen activator (t-PA and u-PA) increase, while the mRNA levels of their inhibitors type 1 and type 2 (PAI-1 and PAI-2) and the urokinase receptor (u-PAR) are also modulated.

The occupancy of the urokinase receptors changes in a striking way during the differentiation process. During the proliferation phase the receptors are mainly occupied, during the initiation of the differentiation phase they are almost completely free, and later during the differentiation process they are nearly fully occupied again. The role of the u-PA receptor in the process was further demonstrated by the inhibition of the differentiation process by the competition of the receptor binding of u-PA, or the removal of the receptor from the cell surface by phospholipase-C treatment.

INTRODUCTION

Differentiation of myogenic stem cells, i.e. satellite cells, is the key process in regeneration of muscle in vivo. This process can be mimicked in vitro using primary cultures of myogenic satellite cells (Campion et al., 1984; Moss et al., 1971; Mauro, 1961, 1979). Differentiation involves the fusion of mononucleated cells to form multinucleated myotubes. Activated satellite cells first proliferate and migrate, align themselves and finally undergo a fusion process to form myotubes (Bischoff, 1974; Yablonka-Reuveni et al., 1987). It has been suggested that the processes of cell migration and fusion could be mediated by localized extracellular proteolysis (Strittwater et al., 1982), a feature common to several other physiological and pathological tissue remodeling conditions such as embryogenesis, neuron development and tumor cell invasion (Danø et al.,1985; Pollanen et al.,1991).

The most thoroughly investigated, and possibly the most widespread, extracellular proteolytic pathway is represented by the activation of the inactive plasminogen to the broad spectrum serine protease plasmin (Danø et al., 1985; Pollanen et al., 1991). Two types of plasminogen activators, tissue-type (t-PA) and urokinase-type (u-PA) are able to activate plasminogen, thus triggering a cascade of proteolytic events which is involved in cell migration and tissue remodeling as originally proposed by Reich (1978). However, the proteolytic cascade appears to be extremely complex in that, in addition to t-PA and u-PA, it involves a variety of regulatory steps like activation of the proenzymes (in

particular pro-urokinase), synthesis of specific inhibitors, receptor binding and surface localization of the plasminogen activating activity (Blasi et al., 1987; Hajjar et al., 1990) as well as of plasminogen itself (Plow and Miles, 1988).

Two plasminogen activator inhibitors, type 1 (PAI-1) and type 2 (PAI-2), have been described (Kruithof, 1988; Sprengers et al.,1987) which act by regulating the enzymatic activity both in solution and associated with the cell surface (Cubellis et al., 1989,1990; Estreicher et al., 1990). Surface association of plasminogen activators is induced by the presence of specific receptors, the u-PA receptor (u-PA-R) being the best characterized (Vassalli et al., 1985; Stoppelli et al., 1985; Blasi, 1988). This receptor has a high affinity and specificity for u-PA and appears to be a key molecule in pro-uPA activation, cell surface proteolytic activity and its regulation as well as for internalization and degradation of u-PA:PAI complexes (Cubellis et al., 1986,1989,1990; Ellis et al., 1989; Estreicher et al.,1990; Pollanen et al., 1990; Stephens et al., 1989).

We have previously shown that during in vitro differentiation of rat skeletal muscle the alignment and fusion of the myogenic cells is accompanied by modification of plasminogen activator activity (Barlovatz et al., 1990). Since the plasminogen activator system in human cells is better characterized at the molecular level, we studied the in vitro differentiation of human satellite cells and correlated the human muscular differentiation process with the regulation of the entire plasminogen activation system, and demonstrate the essential importance of this system, and the u-PA receptor specifically, in the fusion process.

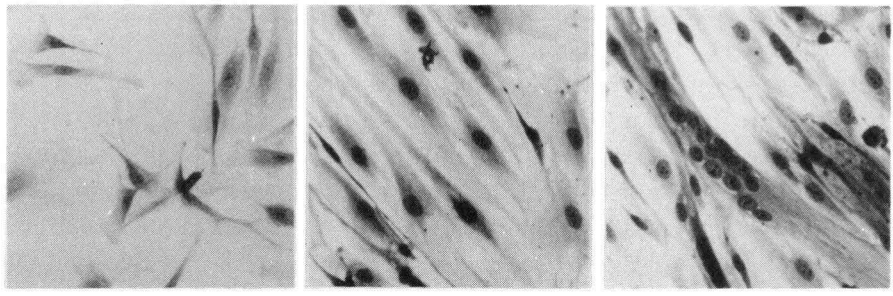
RESULTS

In vitro proliferation and differentiation of myogenic satellite cells.

Myogenic satellite cells from human adult skeletal muscle grow and differentiate in vitro. After the proliferation phase, characterized by randomly organized cells (fig. 1a), myogenic cells align (fig. 1b) and eventually fuse to form myotubes consisting of multinucleated cells (fig. 1c).

The maximum of proliferation is observed between day 7 and day 8 (fig. 2a), and decreases thereafter. The differentiation, expressed as the daily fusion index, starts impressively after day 10, when most of the proliferation phase has already passed (fig. 2b). Differentiation continues after day 12 but can no longer be quantified precisely.

The differentiation process can also be monitored by the use of biochemical markers. As shown in figure 3 α-actin mRNA, used as a control for muscle cell differentiation (Buckingham, 1985) is already detectable under our culture conditions in myogenic cells at day 4, and increases gradually from that time to day 22.



A B C

Figure 1. Morphology of satellite cells during in vitro myogenesis.
Micrograph of satellite cells in culture at day 4 (A), at day 8 (B) when the cells align and the fusion process starts, and at day 22 (C) when multinucleated myotubes are formed, are shown. Cells were stained as described in materials and methods.

Regulation of plasminogen activator activities during differentiation.

t-PA activity can be detected in myogenic cell extracts. It increases steadily starting from day 5 and remains elevated during the differentiation stage (fig. 4a). u-PA activity in the cell extracts increases in a two step manner. A moderate increase is seen from day 7 at the initiation of the differentiation process and a very pronounced increase is observed from day 11 (fig. 4b). The rapid increase in uPA activity from day 11 coincides with the rapid increase in differentiation.

Presence of u-PAR in human myogenic cells, and changes of occupancy during differentiation.

The presence and state of occupancy of u-PAR in human myogenic cells and its regulation during myogenesis have been evaluated by cross-linking and cell binding experiments. As shown in figure 5a human myogenic cells specifically bind ^{125}I-labeled ATF (amino terminal fragment) of u-PA (Stoppelli et al., 1985) forming a 65 kD cross-linked complex. This complex co-migrates with the complex formed in cell extracts of mouse LB6 cells transfected with a human u-PAR cDNA expression vector (Roldan et al.,1990), (fig. 5a, right lane) strongly suggesting that it is the u-PA receptor.

The state of occupancy of the u-PA receptor has been evaluated by measuring the number of receptors before and after mild acid treatment, a procedure that is able to

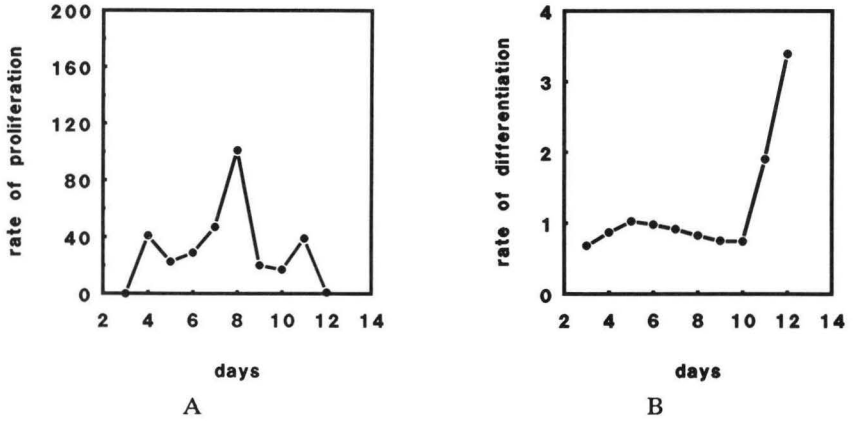


Figure 2. Proliferation and differentiation of satellite cells during in vitro myogenesis. The modulation of the proliferation rate (A) and differentiation rate (B), expressed as daily increase, determined as described in materials and methods, during the in vitro myogenesis process is indicated.

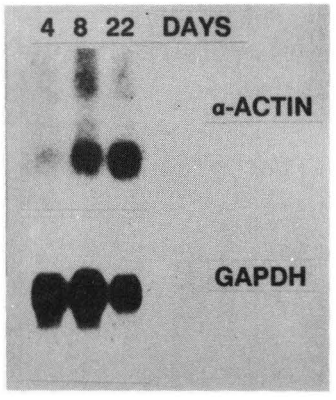
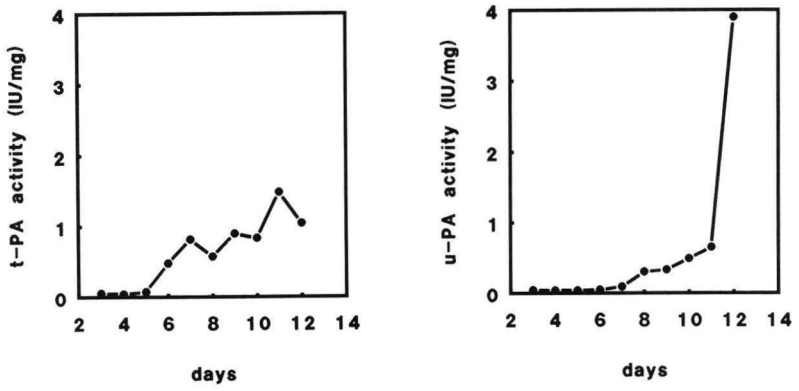


Figure 3. α-Actin mRNA during in vitro myogenesis. 10 μg of total RNA isolated at day 4, 8 and 22 are hybridized with an α-actin probe, as a marker for differentiation. A GAPDH probe was used as an internal standard.

dissociate endogenous u-PA or pro-u-PA from the u-PA receptor (Stoppelli et al., 1986). The occupancy of the u-PAR changes with the state of proliferation and differentiation (fig. 5b). During the proliferation the percentage of free receptors increases from 30% at day 4 to 85% at day 8 at the initiation of the differentiation, while during the differentiation stage at day 12 the percentage of free receptors is decreased to 4%.

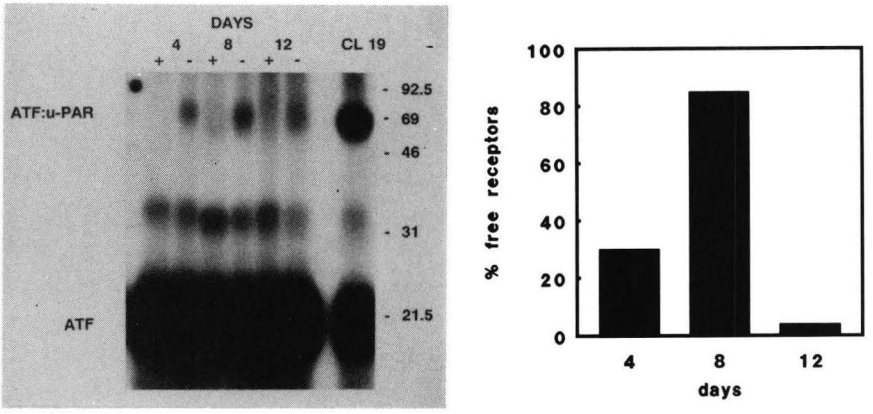
mRNA levels of the components of the plasminogen activation system during differentiation.

To clarify the molecular events leading to an increase in plasminogen activating activity,



A B

Figure 4. Plasminogen activator activity during in vitro myogenesis. The t-PA activity (A) and u-PA activity (B) in cell extracts were determined during the in vitro myogenesis process. Activities are expressed in IU per mg protein.



A B

Figure 5. u-PA receptor during in vitro myogenesis. Binding and cross-linking of ^{125}I-ATFto cell lysates of cultured satellite cells was analyzed in the absence (-) and presence (+) of excess unlabeled ATF (A). Cell lysate of mouse LB6 cells producing the human u-PAR was used as a control for receptor binding of ATF (CL19). The receptor occupancy (B) was analyzed using mild acid treatment and is shown as percentage of the total number of receptors present.

we have studied the levels of the mRNAs of t-PA, u-PA, PAI-1, PAI-2 (figure 6) as well as u-PAR (figure 7) at three moments, day 4, 8 and 22, during the differentiation of human myogenic cells. Day 22 was included because at this point the differentiation was

established. Northern blot hybridization was performed with the appropriate probes, and the mRNA levels compared to that of GAPDH mRNA as an unmodified control. As shown in figure 6, t-PA and u-PA mRNA levels increase during differentiation, in a similar way as the skeletal α-actin mRNA (figure 3). A moderate increase in PAI-1 mRNA is observed at day 8. The PAI-2 signal, absent at day 4, rises to quite high levels at day 8, following a similar pattern as α-actin mRNA. The level of mRNA for both PAI-1 and PAI-2 appears to be somewhat lower at day 22 than that at day 8, although the control GAPDH signal varied also. Finally the level of u-PAR mRNA appears to undergo a change with the length of the differentiation time, being increased at day 8, just prior to the onset of fusion, and subsequently being decreased again at day 22 (figure 7).

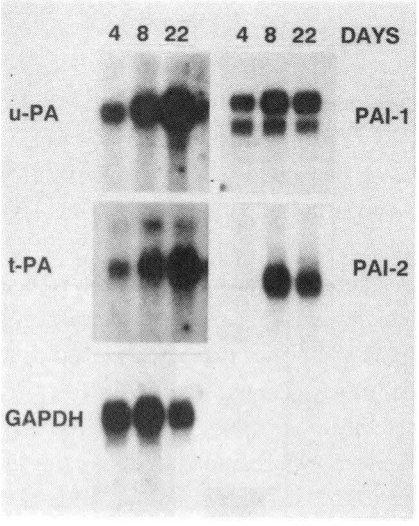


Figure 6. mRNA analysis for plasminogen activators and inhibitors. 10 μg of total RNA isolated from cultures of 4, 8 and 22 days were analyzed using cDNA probes for t-PA, u-PA, PAI-1 and PAI-2. A GAPDH cDNA probe was used as an internal control. Exposure times of the autoradiograms for t-PA and u-PA were 4 days, for PAI-1, PAI-2 and GAPDH one day.

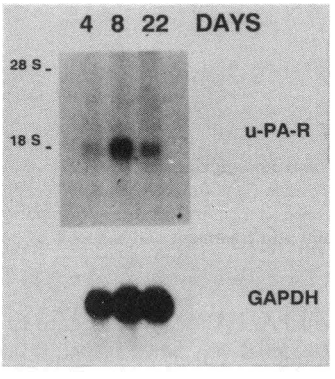


Figure 7. u-PA receptor mRNA analysis. 10 μg of total RNA isolated from cultures of 4, 8 and 22 days was analyzed for the presence of u-PAR mRNA. GAPDH was used as an internal control.

Role of u-PAR during the fusion process.

The previous data demonstrate a coordinate regulation of the entire plasminogen activation system during myotube formation. In particular, the change in receptor occupancy is striking. To analyze the role of surface-bound u-PA activity in the differentiation of myogenic cells in culture, we have used two different approaches to prevent the binding of endogenous u-PA to cell surface receptors. In the first, more specific approach we have incubated cultures of myogenic cells from day 4 on with 50 or 100 ng/ml of the aminoterminal fragment of u-PA (ATF). The extent of differentiation was followed for an additional 6 days, bringing the total to 10 days in culture, the period in which initiation of the differentiation takes place. As shown in figure 8 for control cells the number of nuclei incorporated in myotubes increases from 70 to 2100 nuclei/cm^2 from day 4 to day 10, the largest increase occurring between day 8 and day 10. In the presence of 50 ng/ml ATF, the differentiation is clearly decreased, the number of nuclei incorporated in myotubes at day 10 being decreased to 1514 nuclei/cm^2 (25% inhibition). At 100 ng/ml ATF the inhibition is much clearer, the number of nuclei incorporated in myotubes being decreased to 751 nuclei/cm^2 (65% inhibition). Interestingly, hardly any inhibition occurs in the period between day 4 and day 8, the stage prior to the initiation of differentiation.

A second, less specific, approach to prevent endogenous u-PA binding to the receptor at the cell surface is to treat the cells with phosphatidylinositol dependent phospholipase C (PI-PLC). This treatment releases many surface molecules that are attached to the membrane via a glycolipid anchor including the u-PAR (Ferguson and Williams, 1988; Ploug et al., 1990). As shown in figure 8, treatment of human myogenic cells with 1 U/ml of PI-PLC results in a drastic inhibition of myotube formation. In this case 40% inhibition is observed already at day 6 and day 8 (i.e. 2 and 4 days after addition of PI-PLC), but reaches 90% at day 10.

These results also correlate very well with the observation that the increase in u-PA activity occurs around day 7-8 (fig. 4b).

DISCUSSION

Human myogenic stem cells, when grown in vitro, proliferate and differentiate into multinucleated myotubes. This in vitro differentiation process was monitored morphologically using microscopy (figure 1) and biochemically by the increase in α-actin mRNA (figure 3), a marker for muscle cell differentiation (Buckingham, 1985). The in vitro differentiation of satellite cells (myogenic stem cells) was paralled by an increase in u-PA activity, while at the RNA level the increase in α-actin mRNA correlated with

an increase in u-PA mRNA as well as t-PA mRNA. The time dependent increase in t-PA activity in the cell extracts seemed to parallel the proliferation of the cells.
The mRNA synthesis of both the inhibitors, PAI-1 and PAI-2, as well as the u-PA receptor was also modulated during in vitro differentiation. The mRNA levels were

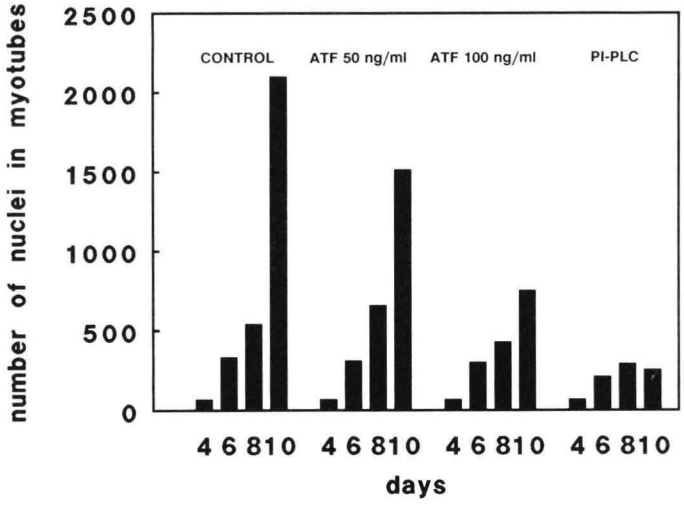


Figure 8. Inhibition of differentiation by ATF or PI-PLC. The number of nuclei incorporated in the myotubes was determined for control cells, cells incubated with 50 ng/ml ATF, cells incubated with 100 ng/ml ATF and cells incubated with 1 U/ml PI-PLC at day 4, 6, 8 and 10.

increased at day 8, the initiation of the differentiation stage, while they were slightly decreased at day 22. The increase from day 4 to day 8 was remarkable, especially for PAI-2.
The fact that all components of the plasminogen activating system are clearly affected during the muscle differentiation process suggests a role for plasminogen activation and generation of the active protease plasmin in the local extracellular proteolytic processes accompanying muscle cell migration and fusion during the formation of myotubes.
Surface localization of the proteolytic activity may be important in cell migration processes, as was suggested by the studies of tumor cell migration (Ossowski, 1988). The u-PA receptor appears to be a key molecule in the cell surface localization of proteolytic activity and its regulation. Binding of pro-u-PA to its receptor increases its activation to active u-PA (Cubellis et al., 1986; Ellis et al.,1989), whereas inactive u-PA:PAI complexes are rapidly removed from the cell surface by internalization and subsequent degradation (Cubellis et al, 1990; Estreicher et al., 1990).
Functional u-PA receptors were demonstrated on the cell surface during all stages of the in vitro myogenesis (figure 5a.). The occupancy of the receptors, however, varied markedly during the myogenesis process (figure 5b.). At day 4, at the beginning of the

proliferation stage, the majority of the receptors was occupied (70%), at day 8, just before the differentiation stage begins, only a small portion of the receptors was occupied (15%), while at day 12, the day the differentiation has started and the last day the differentiation could be quantified, nearly all the receptors were occupied again (96%). The level of the u-PA receptor mRNA varied in a similar way, low at day 4, increased at day 8, and decreased again at day 22, when differentiation was established.

This typical modulation of the u-PA receptor during in vitro myogenesis suggests an important role of the u-PA receptor in this process. To study the role of the u-PAR in more detail, we incubated the differentiating muscle cells either with PI-PLC to remove the receptor from the cell surface, or with the receptor binding domain of u-PA, the ATF fragment, to block the receptor binding of u-PA.

Removal of the u-PA receptor from the cell surface by PI-PLC treatment, which releases the glycolipid anchor of the u-PA receptor from the membrane, inhibited the in vitro myogenesis very potently. Inhibition occurred not only at the beginning of the differentiation stage at day 10, but also in the earlier events at day 6 and 8. From these data it can be concluded that the binding of u-PA to the cell surface receptors appears to be crucial for muscle cell differentiation. The inhibition of muscle cell differentiation by PI-PLC is a phenomenon that has been reported already by (Nameroff et al., 1973, 1976). PI-PLC treatment may have many other effects on the cells besides removal of the u-PAR from the cell surface, as the cleavage of cell surface neuronal cellular adhesion molecules (Knudsen et.al., 1989). A more specific way to study the role of the u-PA binding to the cell surface receptor is to block the u-PA binding to its receptor by competition with the ATF fragment of u-PA.

It was clearly demonstrated in figure 8 that the differentiation process occurring at day 10 was inhibited in a dose dependent way in the presence of ATF, in earlier stages hardly any effect could be observed.

The modulation of the various components of the plasminogen activation system during the in vitro muscle cell differentiation process makes it possible to hypothesize on the interactions between the various components during this process. Especially of interest is the fact that at day 8, just before the differentiation stage, most of the receptors are free, PAI-2 mRNA becomes abundantly present, and u-PAR mRNA is increased. It has been reported before (Cubellis et al.,1990, Estreicher et al., 1990) that receptor bound u-PA is rapidly internalized when complexed to an inhibitor. When PAI-2 levels are increased the internalization of receptor bound u-PA:PAI may induce the u-PA receptor synthesis as well as u-PA synthesis, as is suggested by elevated mRNA levels. The newly formed u-PA receptor, most likely localized at the leading edge of the cell fusion processes, will either be free or occupied with a u-PA:PAI complex and subsequently internalized rapidly. u-PA production is still increasing and will cause that the receptors are occupied during the later stages of the differentiation process.

Treatment with PI-PLC inhibited muscle cell differentiation also in the very early stages of the differentiation process while ATF competition had only effect in the later stages when initiation of the differentiation process occurs. This might be explained by the fact that removal of the u-PA:u-PAR complexes from the cell surface has an effect in all stages of the myogenesis process, while competition of u-PA binding with ATF could be more efficient when the receptors are free. Unfortunately, about the role of t-PA in the myogenesis process little can be speculated from our data.
The involvement u-PA binding to its cell surface receptor in pathological extracellular proteolytic processes, e.g. tumor cell invasion, has been described. Here we show an equally important role of this receptor in a physiological process, i.e. muscle cell differentiation.

MATERIALS AND METHODS

Cell culture

Human muscle cells were cultured according to the method described previously by Blau et al. with a few modifications (Blau et al., 1981; Delaporte et al., 1986; Dodson et al., 1987). Briefly, muscle biopsies, obtained after orthopaedic surgery, were digested in 0.15% pronase in PBS for 1 hr at 37°C. After centrifugation and washing, the cells were seeded at $6x10^4$ cells per flask in Ham F12 medium containing 20% fetal calf serum, 2mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The culture media were routinely changed every 4 days. For all experiments second passage cell cultures were used. Cells were, when indicated, incubated with medium supplemented with aminoterminal fragment of human u-PA (ATF) (50 ng/ml or 100 ng/ml) or with 1 unit/ml phosphatidylinositol dependent phospholipase C (PI-PLC). ATF was a gift of Dr. J. Henkin. PI-PLC from Bacillus cereus was obtained from Boehringer Mannheim (Germany). 24 hours before harvesting the cells fresh serum-free medium (containing 0.1% BSA) was added to the cells. Growth was evaluated by counting trypsinized cells resuspended in PBS on a Malassez haemocytometer or for nuclei counting cells were May-Grunwald Giemsa stained. For each time point the growth (daily increase) was calculated and expressed in terms of rate i.e. number of cells per cm^2 at day n minus the number of cells per cm^2 at day n-1 divided by the number of cells per cm^2 at day n-1. At least 500 cells were counted for each determination.
Differentiation of the muscle cells can be monitored by the formation of the multinucleated myotubes from the satellite cells. Differentiation is expressed in terms of a daily increase in fusion index, analogous as described above for growth. The fusion index is the number of nuclei incorporated in the myotubes expressed as the percentage of total. At least 2000 nuclei were counted for each determination.

Plasminogen activator activity

u-PA activity in cell extracts was determined according the method described by (Drapier et al., 1979) and expressed in International Units (IU) of u-PA activity using a commercial u-PA (CHOAY, lot no. 86018DP, 60000 IU/mg) as a standard. t-PA activity in cell extracts was determined using the method as described by (Angles-Cano, 1986) and expressed in International Units (IU) of t-PA activity using the International Standard preparation (WHO 83.517) as a standard. The chromogenic substrates S2251 and CBS3308 were from KABI (Stockholm, Sweden) and Stago (Paris, France) respectively.

u-PA receptor analysis

The presence of specific urokinase receptors on the cells was determined essentially according to (Nielsen et al., 1988). Briefly, cells were detached from the culture dishes using a rubber policeman and washed twice with PBS. After a moderate acid treatment (0.05 M glycine, pH 3.0) to remove the endogenous u-PA from the receptor, cells were lysed. Cell lysates were incubated with radiolabeled amino terminal fragment of u-PA, ^{125}I-ATF, in the presence or absence of excess unlabeled ATF, and crosslinked with disuccinimidyl suberate (2 mM). Subsequently the samples were analyzed by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) followed by autoradiography using Kodak XAR-5 film and intensifying screens at -70°C.

u-PA binding assay.

Cells were washed with medium or binding buffer (50 mM HEPES, pH 7.0, 1 mg/ml BSA) and incubated with ^{125}I-ATF, either directly or after a mild acid treatment (50 mM Glycine, pH 3.0). The specificity of the assay was determined by addition of an excess of unlabeled ATF. After removal of the supernatant, cells were washed four times in washing buffer (PBS, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mg/ml BSA, pH 7.4) and scraped in PBS. Radioactivity in the cell fraction was determined using a γ-counter.

mRNA analysis

Cells were washed with PBS at 37°C and lysed in 4 M Guanidium thiocyanate, 25 mM sodium citrate, pH 7.5, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol. RNA was isolated according to (Chomczynski and Sacchi, 1987). The total amount of RNA isolated was determined by measuring the OD_{260} assuming that 1 OD_{260} unit is equivalent to 40 μg RNA.

RNA samples were analyzed using electrophoresis on a 1.2% denaturating agarose gel containing 7.5% formaldehyde and hybridization with ^{32}P-labeled cDNA fragments as described before (Quax et al., 1990).

Autoradiograms were prepared using Kodak XAR-5 films and intensifying screens at -70°C.

cDNA probes

For the hybridization experiments the following cDNA fragments were used as probes: a 1.9 kb Bgl II fragment of the human t-PA cDNA (van Zonneveld et al., 1987), a 1.0 kb EcoRI-Pst I fragment of the human u-PA cDNA, kindly provided by Dr. W.D. Schleuning (Medcalf et al., 1988), a 1.2 kb PstI fragment of the human PAI-1 cDNA (van den Berg et al., 1988), a 1.2 kb EcoRI fragment of the human PAI-2 cDNA, kindly provided by Dr. E.K.O. Kruithof (Schleuning et al., 1987), a 0.6 kb BamHI fragment of the human u-PA-R cDNA (Roldan et al., 1990), a 0.2 kb SacI-HindIII fragment of the rat skeletal muscle specific α-actin cDNA, kindly provided by Dr. M. Buckingham (Sassoon et al., 1988), and a 1.2 kb PstI fragment of a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, kindly provided by Dr. R. Offringa (Fort et al., 1988).

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CHAPTER 8

GENERAL DISCUSSION

In this thesis further evidence is given for the fact that the plasminogen activation system, with the active serine protease plasmin as its end product, should be regarded as a general proteolytic mechanism which can be involved in many processes. These processes may be both of a pathological nature, like tumor cell migration and metastasis, or of a physiological nature, like fibrinolysis or cell migration and fusion during muscle cell differentiation.

The general role of the plasminogen activation system is also indicated by the widespread in vivo distribution of the various components of the plasminogen activation system. Although the studies in rats showed that the plasminogen activator t-PA and the inhibitor PAI-1 were mainly detectable in organs containing much vascular tissue, like lung, heart and aorta, in many other tissues various components of the plasminogen activator system were detectable, especially after induction by endotoxin. u-PA was only detected in kidney and lung tissue. The distribution of the various components of the plasminogen activator system may be very distinct. In the liver, only the endothelial cells were found to contain PAI-1 mRNA, and after endotoxin induction, only small amounts of PAI-1 mRNA were found in the liver parenchymal cells. In the aorta the smooth muscle cells of the media contained PAI-1 mRNA, although in the experimental set-up used, the specific contribution of the endothelial cells could not be determined. t-PA appeared to be produced in all layers of the aorta.

As a model system to study the regulation of the plasminogen activator activity in vivo, endotoxin treatment was used. Endotoxin is known to increase plasma PAI activity in rat (Collucci, 1986; Emeis, 1986). Furthermore it was reported that endotoxin could affect the t-PA antigen concentrations in humans (Sufferendini, 1989). Endotoxin treatment of rats decreased the plasminogen activator activity in all the tissues studied (chapter 2). This decrease in plasminogen activator activity was due to an increase in PAI-1, since, a) PAI-1 mRNA levels were increased in all tissues studied (chapter 2), although also a slight increase in t-PA mRNA was detectable in some tissues. b) t-PA concentrations in the aorta layers remained constant, while the PAI activity was modulated after endotoxin treatment. The increase in PAI activity was due to an increase in PAI-1 activity (chapter 3).

The increase in PAI activity was due to an increase in PAI-1 mRNA in nearly all the tissues and cell types studied. After endotoxin induction elevated levels of PAI-1 mRNA were detectable in all tissues studied.

From these studies, as presented in chapter 2 and 3, it can be concluded that the regulation of the plasminogen activator activity after endotoxin treatment occurs at the level of the PAI-1 inhibitor, more precisely by induction of the PAI-1 mRNA.

As a first step in the study of the role of plasminogen activators in tumor development and metastasis, the production of and the regulation of the production of plasminogen activators and their inhibitors in human tumor cells was studied. All tumor cell lines studied produced at least one kind of plasminogen activator. Not only u-PA was detected but also t-PA was detected frequently and in comparable concentrations, as was described previously (Cajot, 1986). Most interestingly, nearly all the cell lines studied produced at least one kind of inhibitor. It is suggested that not only u-PA, but also t-PA and the inhibitors may play a role in tumor associated extracellular proteolysis.

In these tumor cell lines, the plasminogen activator and their inhibitor concentrations appears to be regulated at the mRNA level, but also a second regulatory step at the protein synthesis level appears to occur (chapter 4).

The regulation of the plasminogen activator activity is a complex mechanism. As mentioned in the introduction of this thesis, it may occur at the mRNA level, at the protein synthesis level or even at the level of activation and localization of the enzymes and inhibitors. From the studies in chapter 2, 3 and 4 it can be concluded that regulation of the mRNA levels of the components of the plasminogen activator system is one of the major regulatory mechanisms.

The mRNA levels of plasminogen activators and their inhibitors are determined by the balance between mRNA synthesis (transcription), and turnover. Regulation of both processes has been described for plasminogen activators as well as their inhibitors. Analysis of the transcription regulation of the genes for t-PA and u-PA as well as PAI-1 and PAI-2 revealed many regulatory elements in the promoter sequences of the respective genes (Schleuning, 1990; von der Ahe, 1988; Bell, 1990; Verde, 1988; Riccio, 1988; Andreasen, 1990; Kruithof, 1988). A large variety of agents may affect the transcription of plasminogen activator and plasminogen activator inhibitor genes, like estrogen, glucocorticoids, epidermal growth factor, oncogene products and phorbol esters. A regulation of the mRNA stability is also very likely, since mRNAs of all these genes contain an AU-rich sequence in the 3' untranslated area of the transcripts (van den Berg, 1988). This AU-rich sequence is thought to be involved in the regulation of the mRNA stability (Shaw, 1986; Schuler, 1988).

At the protein synthesis level the translation efficiency but also the protein processing at a postranslational stage may be regulated.

Whether a correlation exists between production of plasminogen activators or their inhibitors and tumor cell associated proteolysis was studied using a set of human melanoma cell lines. These melanoma cell lines differ in their metastatic phenotype in nude mice. It was demonstrated that the production of u-PA as well as PAI-1 and PAI-2, and u-PA mediated degradation of extracellular matrix in vitro correlated with spontaneous metastasis in nude mice (chapter 5). Although a role of u-PA in cellular invasiveness has been demonstrated before (Ossowski, 1983,1988a; Mignatti, 1986) and the involvement of u-PA in metastasis in nude mice after intravenous inoculation has been suggested (Hearing, 1988; Axelrod, 1989) the correlation with PAI production is new. The inhibitors are most likely necessary for a fine tuned regulation of the plasminogen activator mediated extracellular proteolytic processes during tumor cell migration.

A role of the u-PA receptor was also suggested from the data obtained in this metastasis model. Although all melanoma cell lines in vitro could bind u-PA, in vivo mainly the subcutaneous tumors derived from the metastasizing cell lines showed a clear u-PA binding. The importance of cell surface bound u-PA in cellular invasiveness and metastasis was already previously suggested (Ossowski, 1988b; Hearing, 1988).

Although a correlation was found, conclusive evidence for the role of the u-PA system in metastasis could not be obtained in our study. To study the role of the separate components of the u-PA system, u-PA and u-PA receptor, in tumor cell associated extracellular proteolysis, a complementation system was developed. The non-proteolytic mouse L-cells were transfected with either the human u-PA or the human u-PAR gene. Analysis of the proteolytic capacity of these cells as well as co-cultures of these cells, confirmed the role of u-PA in extracellular proteolysis. Transformation of mouse-L cells with u-PA was sufficient to change them into proteolytically active cells. Cajot et al. (Cajot, 1989) have shown that transformation of mouse L-cells with u-PA not only gave these cells the capacity to degrade an extracellular matrix in vitro, but also changed them into invasively growing cells. Moreover, our studies showed that the u-PA receptor has also an important function in extracellular proteolysis.

Binding of u-PA to its cell surface receptor enhances the proteolytic activity, especially at lower u-PA concentrations. The direct effect of u-PA in extracellular proteolysis was only observed in cells producing relatively high amounts of u-PA.

When u-PA is present at lower concentrations, enhancement of proteolytic activity occurs probably because of the localization of the plasminogen activation process at the cell surface. This cell surface localization is necessary to achieve an effective activation of the pro-enzyme of u-PA, pro-u-PA, by plasmin in the presence of inhibitors of plasmin or plasminogen activators. Competition experiments of receptor binding of u-PA, as well as removal of the receptor from the cell surface, inhibited the extracellular proteolysis. From the concomitant binding of plasminogen and (pro-)u-PA to their respective cell

surface receptors (Plow, 1986; Stephens, 1989), it was suggested that the whole process of plasminogen activation takes place more effectively because of the appropriate localization of both components in their proximity. The causal involvement of u-PA and especially the enhancing role of receptor binding of u-PA in extracellular proteolysis, as demonstrated (chapter 6), might provide physiological significance to the previous findings indicating an important role for the u-PA receptor in cellular invasiveness (Ossowski, 1988b) and laminin degradation (Schlechte, 1989).

The role of the plasminogen activator system in extracellular proteolysis in the physiological processes of muscle cell differentiation appeared to be very similar to that in pathological processes, like tumor cell invasion. The involvement of the plasminogen activation system in muscle cell differentiation was demonstrated by the complex modulation of the plasminogen activator activity during in vitro myogenesis. The u-PA activity increases during in vitro myogenesis in parallel with the state of differentiation of the myogenic stem cells, while t-PA seems to increase in parallel with the state of proliferation. The inhibitors and u-PA receptor are also modulated during this process. The complex regulation of the various components of the plasminogen activator system during this process of in vitro myogenesis makes it possible to study the interactions between the various components at the different time points in the process. For the u-PA system the cell surface localization appears to be most important since competition experiments for u-PA receptor binding or removal of the receptor from the cell surface inhibited the differentiation process markedly. The function of the changes in t-PA activity in myogenesis process is not yet fully understood.

From this thesis it can be concluded that the plasminogen activator system should be regarded as a general mechanism involved in many extracellular proteolytic events, since it not only plays a role in pathological processes, like tumor development and metastasis and endotoxin related reactions, but also in more physiological processes like cell migration and myogenesis. However, it should be realized that also other proteolytic systems, such as the metalloproteinase system, may play an important role in the regulation of extracelullar proteolysis. A better understanding of the exact function of the separate components of the plasminogen activation system as well as the interaction of these components may lead in the end to a way to interfere in many processes, including tumor development and metastasis.

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SUMMARY

This thesis has been focused on the role of the plasminogen activation system in extracellular proteolytic processes, both physiological and pathological, involving cell migration and tissue remodeling.

First the distribution of the various components of the plasminogen activation system in vivo was studied. The occurrence of plasminogen activators and their inhibitors in vivo appeared to be widespread. It was demonstrated in the rat that plasminogen activator t-PA and the plasminogen activator inhibitor type 1 (PAI-1) were present in many tissues. The highest mRNA concentrations were found in the organs contaning much vascular tissue, such as lung, heart and aorta (chapter 2 and 3). Despite the widespread occurrence of plasminogen activators and inhibitors in different tissues and organs, there is a distinct distribution of plasminogen activator inhibitor in the specific cell types of these tissues and organs studied. In the liver, PAI-1 is mainly detected in the endothelial cells (chapter 2), while in the aorta the smooth muscle cells were shown to produce PAI-1 (chapter 3). Endotoxin treatment was used as a model system to study the regulation of plasminogen activator activity in vivo in the rat. The decrease in plasminogen activator activity after endotoxin treatment is due to an increase in PAI-1 mRNA in nearly all the tissues studied. The PAI activity not only increased in plasma, but the PAI activity was also locally increased within the rat aorta tissue.

A role of the plasminogen activator system in tumor development was suggested previously based on the generally increased concentrations of plasminogen activators in tumor tissues or tumor cell lines. In chapter 4, the plasminogen activator and inhibitor production and its regulation by twenty two different human tumor cell lines were determined. All of the cell lines studied produced at least one type of plasminogen activator and nearly all of them produced also at least one type of inhibitor. Remarkably, t-PA was found as frequently as u-PA. Regulation of the plasminogen activator and the inhibitor production occurred not only at the mRNA level, as was demonstrated in chapter 2 and 3 for in vivo regulation, but also a second regulatory step at the protein synthesis level was demonstrated to occur in these cell lines in vitro (chapter 4).

In a set of human melanoma cell lines the production of u-PA, PAI-1 and PAI-2, and u-PA mediated extracellular matrix degradation in vitro was found to correlate with the development of spontaneous lung metastasis in nude mice after subcutaneous inoculation of the cells. t-PA production and t-PA mediated extracellular matrix degradation did not correlate with a spontaneous metastasis in nude mice. Differences in the occupation of the u-PA receptor in vivo suggested also a role of this receptor in the metastasis process (chapter 5).

During the metastasis process tumor cells have to migrate through the extracellular matrix several times. The matrix is degraded proteolytically during this process. The causal involvement of the u-PA system in extracellular proteolysis was demonstrated by cloning into the non-proteolytic mouse L-cells the genes for human u-PA or u-PA receptor. After transformation with u-PA these cells gained the ability to degrade extracellular matrix in vitro. In co-culturing experiments of receptor producing and u-PA producing cells it was found that binding of u-PA to the cell surface receptor enhances the proteolytic activity. This enhancement could be explained by a more efficient activation of pro-u-PA bound to its cell surface receptor. Blocking of the receptor binding or removal of the receptor from the cell surface inhibited the enhancing effect (chapter 6).

The plasminogen activator system is also involved in non-tumor cell associated proteolytic processes. During muscle cell differentiation in vitro a complex regulation of the plasminogen activator activity takes place. Not only u-PA and t-PA mRNA levels and activities are increased, but also the PAI-1, PAI-2 and u-PA receptor mRNA levels are modulated during the process. The role of u-PA binding to its cell surface receptor appears to be especially important also in this case, since blocking of u-PA binding to its receptor or removal of the receptor from the cell surface markedly inhibited the in vitro muscle cell differentiation (chapter 7).

It can be concluded that the plasminogen activation system with the complex regulation of the production of and interactions between its various components, should be regarded as a general proteolytic mechanism involved in many physiological and pathological processes.

SAMENVATTING

Het in dit proefschrift beschreven onderzoek is gericht op de rol van het plasminogeen activatie systeem in extracellulaire proteolytische processen, zowel fysiologisch als pathologisch, waarbij celmigratie en weefsel hermodellering betrokken zijn.

Als eerste is de verdeling van de verschillende componenten van het plasminogeen activatie systeem in vivo bestudeerd. Het voorkomen van plasminogeen activatoren en hun remmers in vivo blijkt wijdverspreid te zijn. Het is aangetoond dat in de rat weefseltype plasminogeen activator (t-PA) en plasminogeen activator remmer type 1 (PAI-1) voorkomen in de meeste weefsels. De hoogste mRNA concentraties werden gevonden in die organen welke veel vaatweefsel bevatten, zoals long, hart en aorta (hoofdstuk 2 en 3). Ondanks het wijdverspreide voorkomen van plasminogeen activatoren en remmers in de verschillende weefsels en organen, is er toch een specifieke verdeling van plasminogeen activator remmer over de verschillende celtypen van de bestudeerde weefsels en organen. In de lever wordt PAI-1 voornamelijk aangetoond in de endotheel cellen (hoofdstuk 2), terwijl in de aorta productie van PAI-1 door gladde spiercellen werd aangetoond (hoofdstuk 3).

Endotoxine behandeling is gebruikt als een model systeem om de regulatie van de plasminogeen activator activiteit in vivo in de rat te bestuderen. De afname in plasminogeen activator activiteit na endotoxine behandeling wordt veroorzaakt door een toename in PAI-1 mRNA in bijna alle bestudeerde weefsels. De PAI activiteit is niet alleen verhoogd in plasma, maar ook was de PAI activiteit lokaal verhoogd in het rat aorta weefsel.

Een rol voor het plasminogeen activator systeem in tumor ontwikkeling is al eerder gesuggereerd op grond van de in het algemeen verhoogde concentraties van plasminogeen activatoren in tumor weefsel en tumor cellijnen. De productie van plasminogeen activatoren en hun remmers en de regulatie van deze productie in tweeëntwintig verschillende humane tumor cellijnen is bepaald in hoofstuk 4. Alle bestudeerde cellijnen produceerden minstens één type plasminogeen activator en bijna allemaal produceerden tevens minstens één type remmer. Het is opvallend dat t-PA even vaak gevonden werd als u-PA. De regulatie van de plasminogeen activator en de remmer productie vindt niet alleen plaats op mRNA niveau, zoals in hoofdstuk 2 en 3 werd aangetoond voor in vivo regulatie, maar ook werd in deze cellijnen in vitro het voorkomen van een tweede regulatie stap op het niveau van de eiwit synthese aangetoond (hoofdstuk 4).

In een set humane melanoma cellijnen werd gevonden dat de productie van u-PA, PAI-1 en PAI-2, en u-PA gemedieerde extracellulaire matrix degradatie in vitro correleerde

met de vorming van spontane long metastase in naakte muizen na onderhuidse injectie van de cellen. t-PA productie en t-PA gemedieerde extracellulaire matrix degradatie correleerde niet met spontane metastase in naakte muizen. Verschillen in bezetting van de u-PA receptor in vivo suggereerde tevens een rol voor deze receptor in het metastase proces (hoofdstuk 5).

Gedurende het metastase proces passeren tumor cellen verschillende malen de vaatwand. Ze zullen daarbij door de extracellulaire matrix migreren. De matrix wordt gedurende dit proces proteolytisch afgebroken. De causale betrokkenheid van het u-PA systeem bij extracellulaire proteolyse werd aangetoond door in niet-proteolytisch muize L-cellen de genen voor humaan u-PA of u-PA receptor te cloneren. Na transformatie met u-PA waren deze cellen in staat om in een in vitro model extracellulaire matrix af te breken. Gebruikmakend van co-culture experimenten van receptor producerende en u-PA producerende cellen werd gevonden dat binding van u-PA aan de celoppervlakte receptor de proteolytische activiteit kan versterken. Dit versterkende effect kan verklaard worden door een efficiëntere activatie van pro-u-PA wanneer het gebonden is de celoppervlakte receptor. Blokkeren van de receptor binding of verwijderen van de receptor van het celoppervlakte remt dit versterkende effect (hoofdstuk 6).

Het plasminogen activator systeem is ook betrokken bij niet-tumorcel-geassocieerde proteolytische processen. Gedurende spiercel differentiatie in vitro vindt een complexe regulatie van de plasminogeen activator activiteit plaats. Niet alleen nemen de u-PA en t-PA mRNA niveaus en activiteiten toe, tevens worden de PAI-1, PAI-2 en u-PA receptor mRNA niveaus gedurende dit proces gemoduleerd. De rol van u-PA binding aan zijn celoppervlakte receptor blijkt ook in dit geval bijzonder belangrijk te zijn, aangezien het blokkeren van de receptor binding van u-PA of verwijderen van de receptor van het celoppervlakte de spiercel differentiatie in vitro aanzienlijk remt (hoofdstuk 7).

Het mag geconcludeerd worden dat het plasminogeen activatie systeem, met de complexe regulatie van de productie van en de interactie tussen zijn verschillende componenten, beschouwd moet worden als een algemeen proteolytisch mechanisme dat betrokken is bij vele fysiologische en pathologische processen.

ACKNOWLEDGEMENT

I would like to express my gratitude to all the colleagues at the Gaubius Institute for their support and encouragement and the very pleasant atmosphere, both scientific and social, in which the studies involved in this thesis were carried out.

I am indebted to Jacoline Weening-Verhoeff, Ria van den Hoogen and René van Leeuwen for their excellent technical assistance.

I would like to mention especially Jan Verheijen for the many fruitful and inspiring discussions we had.

Furthermore, I would like to mention: Sjef Emeis, Kees Kluft, Hans Princen, Teake Kooistra, Victor van Hinsbergh, Johan Kuiper and Goos van Muijen for the discussions and collaborations;

Leif Lund, Keld Danø, Georgia Barlovatz-Meimon and Francesco Blasi for the very stimulating collaboration and their hospitality during my visits;

Clara Horsting, Marisa Horsting and Ankie Verwey for their secretarial assistance during the preparation of the manuscripts and literature data base;

Jaap Koopman, Jos Grimbergen, Arjen Bakker, Teresa Padro, Peter de Knijff, Elly de Wit, Richard Prevost, Piter Bosma and Eva van den Berg for their technical and mental support.

Last but not least, I am very grateful to my parents, who made my studies possible and stimulated me all the way, and to you, Rafaëlle, for your everlasting encouraging support and for all your patience and understanding.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 4 mei 1961 te Geleen. Hij volgde de middelbare schoolopleiding aan de scholengemeenschap Sint Michiel te Geleen en rondde deze in 1979 met het behalen van het Gymnasium B diploma succesvol af.
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Sinds oktober 1986 is hij verbonden aan het Gaubius Instituut TNO te Leiden. Aldaar werd binnen de afdeling Fibrinolyse en Proteolyse, onder begeleiding van Dr. J.H. Verheijen, het in dit proefschrift beschreven onderzoek uitgevoerd. Dit onderzoek zal worden voortgezet in het kader van een door het Koningin Wilhelmina Fonds (KWF) gesubsidieerd onderzoeksproject.