

Membrane associated proteases and their inhibitors in tumour angiogenesis

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Abstract: Cell surface proteolysis is an important mechanism for generating biologically active proteins that mediate a range of cellular functions and contribute to biological processes such as angiogenesis. Although most studies have focused on the plasminogen system and matrix metalloproteinases (MMPs), recently there has been an increase in the identification of membrane associated proteases, including serine proteases, ADAMs, and membrane-type MMPs (MT-MMPs). Normally, protease activity is tightly controlled by tissue inhibitors of MMPs (TIMPs) and plasminogen activator inhibitors (PAIs). The balance between active proteases and inhibitors is thought to determine the occurrence of proteolysis in vivo. High concentrations of proteolytic system components correlate with poor prognosis in many cancers. Paradoxically, high (not low) PAI-1 or TIMP concentrations predict poor survival in patients with various cancers. Recent observations indicate a much more complex role for protease inhibitors in tumour progression and angiogenesis than initially expected. As knowledge in the field of protease biology has improved, the unforeseen complexities of cell associated enzymes and their interaction with physiological inhibitors have emerged, often revealing unexpected mechanisms of action.

Abbreviations: ADAMs, a disintegrin and metalloproteinase; bFGF, basic fibroblast growth factor; GPI, glycosyl phosphatidylinositol; MMP, matrix metalloproteinase; MT, membrane-type; PAI, plasminogen activator inhibitor; SP, serine protease; TIMP, tissue inhibitor of matrix metalloproteinases; TTSP, type II transmembrane domain serine protease; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; VEGF, vascular endothelial growth factor.

Angiogenesis, the formation of new blood vessels from pre-existing ones, is essential for sustained tumour growth beyond a critical size of 1-2 mm. Moreover, increased angiogenesis promotes tumour cell penetration into the circulation and thereby metastatic dissemination. Tumour vessels can develop through different mechanisms, including sprouting or intussusception from pre-existing vessels, the mobilisation of circulating endothelial precursors from the bone marrow, and the recruitment of lymphatic vessels (lymphangiogenesis).¹ Different proteolytic enzymes, including serine proteases (SPs) and matrix metalloproteinases (MMPs), have been implicated in angiogenesis.² The human genome sequence has revealed more than 500 genes encoding proteases or protease-like products,³ so that many exciting discoveries about the functions of proteases in physiological and neoplastic processes can be expected in the near future.

"Recent information has underlined the importance of cell surface proteases, their receptors/activators, and their inhibitors during angiogenesis"

Initially, the basic idea for the involvement of SPs and MMPs during cancer progression was that the degradation of extracellular matrix components should contribute to different events, such as provisional matrix remodelling, basement membrane breakdown, and cell migration and invasion. In addition to degrading extracellular matrix components, proteinases have also been implicated in the activation of cytokines, and in the release of growth factors sequestered within the extracellular matrix.⁴⁻⁶ Recent information has underlined the importance of cell surface proteases, their receptors/activators, and their inhibitors during angiogenesis. New membrane associated proteases have been identified and include: type I and type II transmembrane SPs, membrane-type MMPs (MT-MMPs), and ADAMs (a disintegrin and metalloproteinase). In this review, we will use selected examples to illustrate the influence of cell surface proteolysis and the resulting alteration of the pericellular microenvironment on the tumoral angiogenic process. This review will also delineate the unexpected role of physiological inhibitors of membrane associated proteases such as plasminogen activator type 1 (PAI-1) and tissue inhibitors of MMP-2 (TIMP-2).

PROTEOLYTIC SYSTEMS INVOLVED IN CELL SURFACE PROTEOLYSIS

Pericellular proteolysis was initially associated with the classic plasminogen-plasmin system and then extended to at least three new classes of proteases: MT-MMPs, ADAMs, and membrane anchored SPs (figs 1 and 2). These cell surface proteases are integral membrane proteins of the plasma membrane or are anchored to the

membrane through a glycosyl phosphatidylinositol (GPI) linkage.

Figure 1: Membrane-type metalloproteases (MT-MMPs) are associated with the plasma membrane through a transmembrane domain (TM) or a glycosyl phosphatidylinositol (GPI) link. ADAMs (a disintegrin and metalloprotease) contain a transmembrane domain. EGF, epidermal growth factor; Pro, prodomain.

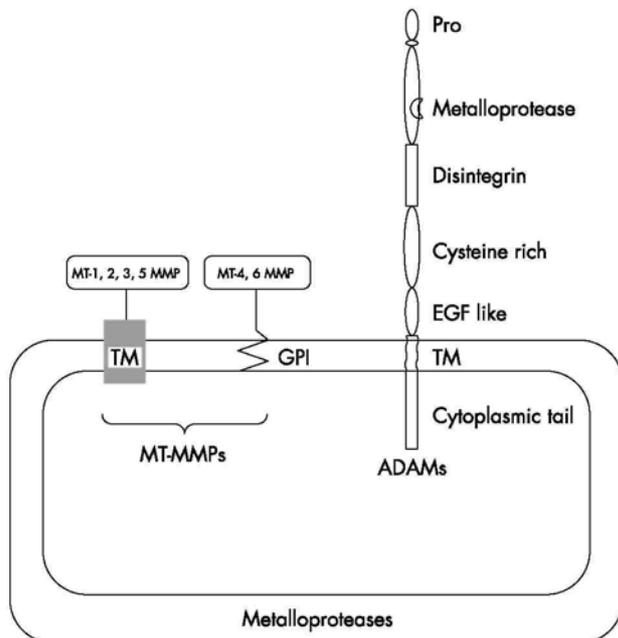
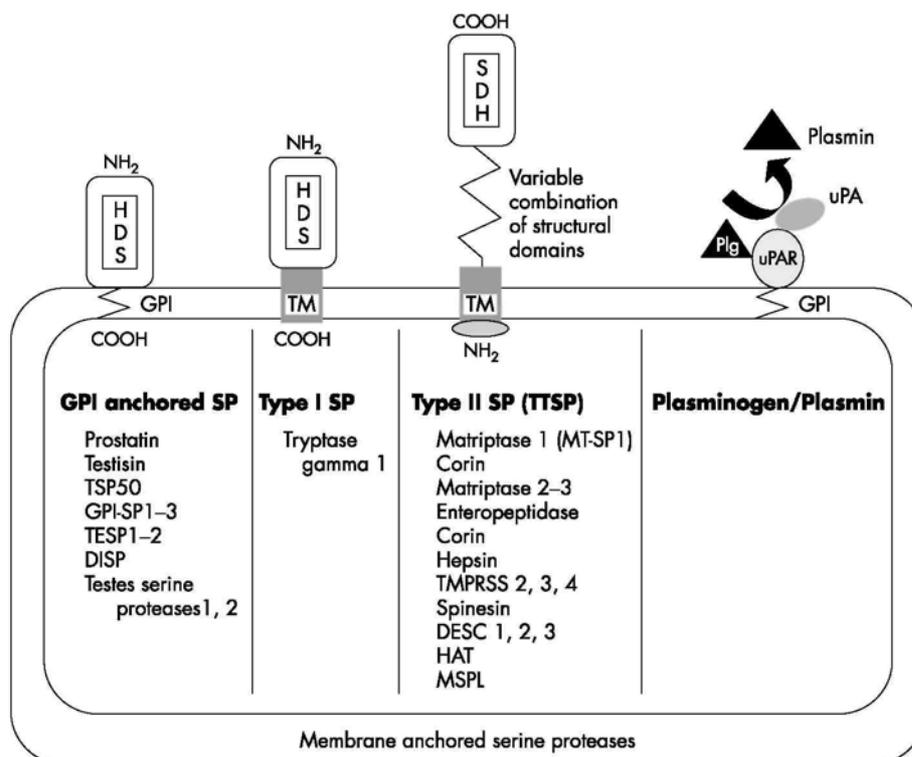


Figure 2: Membrane anchored serine proteases (SPs) are linked to the plasma membrane through a glycosyl phosphatidylinositol anchor (GPI anchored SP), a type I transmembrane domain (TM; type I SP), or a type II TM (type II SP or TTSP). The urokinase plasminogen activator (uPA) receptor (uPAR) containing a GPI link participates in the activation of uPA, leading to the conversion of plasminogen (Plg) into plasmin at the cell surface.

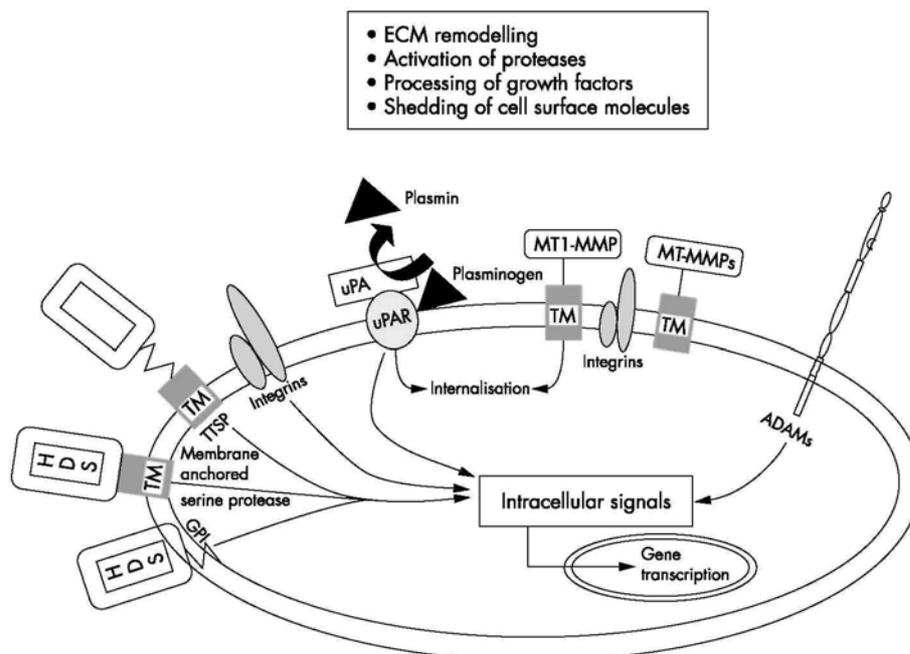


Plasminogen-plasmin system

The plasminogen-plasmin system⁷⁻¹² plays a major role in physiological and pathological processes. It is composed of an inactive proenzyme, plasminogen, which can be converted to plasmin by two SPs: urokinase plasminogen activator (uPA) and tissue-type plasminogen activator. This system is controlled at the level of the plasminogen activators by plasminogen activator inhibitors (PAI-1 and PAI-2), and at the level of plasmin by 2 antiplasmin. uPA binds to a cell surface GPI anchored receptor (uPAR) and controls pericellular proteolysis (fig 2).^{13,14} Plasmin displays a broad spectrum of activity and can degrade several glycoproteins (laminin, fibronectin), proteoglycans, and fibrin to activate pro-MMPs and to activate or release growth factors from the extracellular matrix (latent transforming growth factor β , basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF)⁴ (fig 3)). Studies of knockout mice have revealed that one fundamental role of plasminogen/ plasmin in vivo is fibrinolysis.¹³ Pathological manifestations of plasminogen deficiency, such as impaired wound healing, can be rescued if fibrinogen deficiency is genetically superimposed.¹³ However, given the broad spectrum of activity of plasmin described above, it is conceivable that in addition to fibrin, multiple targets of plasmin are biologically relevant in vivo during physiological and pathological processes. Together with uPAR and PAI-1, uPA is involved in mitogenic, chemotactic, adhesive, and migratory cellular activities.¹⁴¹⁶⁻²¹ The uPA-uPAR complex interacts with vitronectin, a multifunctional matrix glycoprotein and with $\beta 1$ and $\beta 3$ integrins, thereby participating in cell anchoring and migration.^{16, 22, 23} In addition, despite the lack of a transmembrane domain, uPAR colocalises with caveolin, which can bind signalling molecules and stimulate signal transduction through the uPAR.^{19, 22, 24}

“Plasmin displays a broad spectrum of activity and can degrade several glycoproteins, proteoglycans, and fibrin to activate pro-matrix metalloproteases and to activate or release growth factors from the extracellular matrix”

Figure 3: Metalloproteases (MT-MMPs, ADAMs), membrane anchored serine proteases (SPs), and the plasminogen/plasmin system are adequately positioned at the plasma membrane to participate in a cascade of protease activation, to activate receptors, growth factors, and cytokine/chemokines, to shed cell surface molecules, and to participate in extracellular matrix (ECM) remodelling. The presence of a cytoplasmic domain may endow these proteins with the capacity to interact with the cytoskeleton and/or with intracellular signalling molecules, thereby influencing cell shape, differentiation, migration, and gene transcription. ADAMs, a disintegrin and metalloproteinase; GPI, glycosyl phosphatidylinositol; MT-MMP, membrane-type matrix metalloproteinase; TM, transmembrane domain; TTSP, type II transmembrane domain serine protease; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.



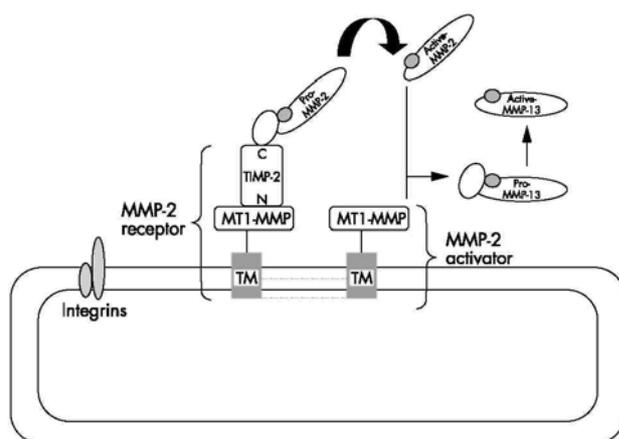
MT-MMPs

MMPs comprise a broad family of 24 zinc binding endopeptidases that degrade extracellular matrix components

and process bioactive mediators. This family has been recently described in excellent reviews.²³⁻²⁸ MMP activity is tightly controlled by endogenous inhibitors, such as $\beta 2$ macro-globulin, and specific MMP inhibitors, the TIMPs. TIMP1, TIMP2, TIMP3, and TIMP4 reversibly inhibit MMPs in a 1: 1 stoichiometric fashion.²³ The different TIMPs differ in tissue specific expression, their ability to inhibit various MMPs, and their capacity to interact with pro-MMPs.²³⁻²⁹ In addition, RECK (reversion induction cystein rich protein with kazal motif) is the only known membrane bound MMP inhibitor.³⁰ It has been reported to contribute to tumour development and angiogenesis.³¹

Although most MMPs are secreted as soluble enzymes into the extracellular milieu, most of the newly identified MMPs are MT-MMPs, which activate latent MMPs (MMP-2) and degrade some extracellular matrix proteins. These MT-MMPs are associated with the cell surface by at least three distinct mechanisms, namely²⁸: (a) a type I transmembrane domain for MT1, MT2, MT3, and MT5 MMPs³²⁻³⁴; (b) a GPI linkage for MT4 and MT6 MMPs (fig 1); and (c) a type II transmembrane domain for MMP23/cystein array MMP. MT1-MMP (MMP-14) is the prototypic member of the MT-MMPs and its expression has been associated with various pathophysiological conditions.³³ It is thought to be the main activator of pro-MMP-2, through the formation of a complex involving MMP-2, its inhibitor TIMP2, MT1-MMP, and $\alpha_v\beta_3$ integrin (fig 3).³⁶⁻³⁹ Although MT2-MMP activates MMP-2 in a TIMP2 independent pathway,⁴⁰ the TIMP2 requirement for this activation process by MT3-6-MMPs remains to be established. In addition to this activator function, MT1-MMP activates pro-MMP-13⁴¹ and displays a broad spectrum of activity against various matrix components.⁴²⁻⁴⁷ However, it has substrates that extend beyond extracellular matrix components, such as cell surface molecules including CD44,⁴⁸ pro α_v integrin,³⁹ and transglutaminase.⁴⁹ Like other MMPs, the activity of MT-MMP is regulated at three main levels: transcription, proenzyme activation, and inhibition.⁵⁻²⁸ The shedding of MT-MMP appears to be an additional way to control enzyme localisation and activity.⁵⁰ In addition, endocytosis emerges as a main mechanism regulating at least MT1-MMP activity.⁵¹⁻⁵³

Figure 4: Activation of pro-MMP-2 and pro-MMP-13 by MT1 -MMP. A model for the activation of pro-MMP-2 has been proposed in which the catalytic domain of MT1 -MMP binds to the N-terminal portion of TIMP-2 (N), leaving the TIMP-2 C-terminal region (C) available for binding to the haemopexin-like domain of pro-MMP-2.³⁶⁻⁵⁴ This ternary complex (MMP-2 receptor) is believed to localise pro-MMP-2 close to a TIMP-2 free MT1 -MMP molecule (MMP-2 activator) able to initiate the activation of pro-MMP-2. This may be favoured by MT1 -MMP oligomerisation through the extracellular domain and the intracytoplasmic tail (dotted lines). MT2-MMP does not require TIMP-2 to activate MMP-2 and it is not known whether the other MT-MMPs form a complex with TIMP-2. Integrins such as $\alpha_v\beta_3$ might also form part of this activation cascade by interacting with MT1 -MMP. A third focus of activation probably involves TTSP, because MT-SP1 has been reported to activate uPA. MMP, matrix metalloproteinase; MT, membrane-type; SP, serine protease; TIMP, tissue inhibitor of matrix metalloproteinases; TTSP, type II transmembrane domain serine protease; uPA, urokinase plasminogen activator.



A disintegrin and metalloproteinases (ADAMs)

ADAMs comprise a family of at least 24 multifunctional membrane proteins.⁵⁵⁻⁵⁸ Structurally related to snake venom metalloproteinases, they display a complex domain organisation consisting of: (a) a prodomain using a cystein switch mechanism similar to that of MMPs; (b) a metalloproteinase domain endowing (or not endowing) the enzyme with proteolytic activity; (c) a disintegrin-like domain mediating cell-cell interaction via integrins; (d) a cystein rich region that may be involved in cell fusion; (e) an epidermal growth factor-like domain; (f) a

transmembrane region; and (g) a cytoplasmic tail (fig 1). In some ADAMs, particularly those with protease activity, the cytoplasmic tail contains a potential phosphorylation site, suggesting potential signalling activity.⁵⁹ Originally associated with reproductive processes, such as spermatogenesis and sperm-egg fusion, more recently they have been linked to other biological processes, such as cell migration and adhesion, and activation of signalling pathways by shedding of membrane bound cytokines and growth factors (fig 4).^{59,60} The first well described ADAM, TACE (tumour necrosis factor α converting enzyme; ADAM17) is essential for the proteolytic release or activation of growth factors and cytokines, including all epithelial growth factor ligands and tumour necrosis factor α , in addition to the shedding of ligands for cell surface receptors.⁶¹ Therefore, these multifunctional molecules are thought to play key roles in different steps of cancer progression. Accordingly, some ADAMs are overexpressed in cancers,⁶²⁻⁶⁴ and their activity can be blocked by TIMPs or synthetic MMP inhibitors.⁶³ ADAM15 contains a unique RGD sequence in its disintegrin domain, which specifically mediates adhesion to $\alpha_v\beta_3$ integrin.⁶⁶ It is therefore conceivable that some ADAMs, through their interaction with key integrins or their proteolytic activity, contribute to the angiogenic process.

"Recently ADAMs have been linked to other biological processes, such as cell migration and adhesion, and activation of signalling pathways by shedding of membrane bound cytokines and growth factors"

Membrane anchored SPs

Recently, rapidly expanding subgroups of SPs have been recognised that are associated with the plasma membrane. These membrane anchored SPs are linked either through a C-terminal transmembrane domain (type I SP), via a GPI linkage (GPI anchored SP), or via an N-terminal transmembrane domain with a cytoplasmic extension (type II transmembrane SP or TTSP) (fig 2).^{67,68} The GPI anchored SPs and type I SPs both contain a hydrophobic domain at their C-terminus and are very similar in length, ranging from 310 to 370 amino acid residues. The GPI anchored proteases may be involved in the dynamic microenvironment of lipid rafts, participating in transduction complexes. The TTSPs share several structural features: an N-terminal cytoplasmic domain of 20 to 160 amino acids, a type II transmembrane sequence, a central region of variable length with modular structural domains, and a C-terminal catalytic region with all the features of SPs.^{57,69} To date, at least 15 TTSPs have been described and are listed in fig 2.⁶⁷⁻⁷²

Membrane anchored SPs are interesting because of their potential capacity to participate in the matrix remodelling associated with cancer.⁶⁹ Their cytoplasmic tail suggests that at least some of them function in intracellular signal transduction. In addition, crosstalk between different proteolytic systems is emphasised by the ability of MT-SP1 to activate uPA.^{73,74} Although their functions are still unclear, they probably contribute to cancer progression because they are overexpressed in several types of cancer.^{67,68,75-81} Matriptase/MT-SP1 has been implicated in tumour growth and metastasis in murine models of prostate cancer.^{82,83} Prometastatic effects have been associated with the stabilisation of active matriptase-1 by glycosylation.⁸⁴ Recently, several TTSPs have been reported to be expressed during microvascular endothelial cell morphogenesis,⁸³ suggesting that they have a function in angiogenesis. Membrane anchored SPs probably have complex functions because the upregulation and downregulation of their expression has been associated with cancer progression.⁶⁷ Further studies are required to elucidate the individual functions of each membrane anchored SP.

ROLE OF THE DIFFERENT PROTEOLYTIC SYSTEMS IN TUMORAL ANGIOGENESIS

A link between the plasminogen system, MMPs, and cancer has been established through extensive studies of tumour biology in both human tissues and animal models.^{10,12,86-88} Although it is anticipated that ADAMs and TTSPs play a role in tumour progression and angiogenesis this has yet to be demonstrated. Mice deficient in one of the plasminogen system components or one MMP or inhibitor have been generated,^{5,89-93} and have in some cases revealed unanticipated roles for previously characterised proteinases or their inhibitors.^{5,89-94}

Role of the plasminogen-plasmin system in tumoral angiogenesis and paradoxical functions of PAI-1

A contribution of the plasminogen-plasmin system to tumour progression is suggested by the following: (a) increased expression of uPA, uPAR, and PAI-1 in various tumours, (b) the use of antisense mRNA, and (c) the administration of natural or synthetic serine proteinase inhibitors, uPA antagonists, or antibodies.^{2,89} This is further supported by the delayed angiogenesis seen recently in plasminogen deficient mice in an in vitro model of aortic rings embedded in a collagen gel,⁹⁵ in addition to an in vivo model of malignant keratinocyte transplantation.⁹⁶ Accordingly, loss of either plasminogen activator or plasminogen was shown to reduce T241 fibrosarcoma^{97,98} and carcinoma^{97,98} tumour growth. In contrast, no difference in tumour growth was seen in a comparative study of control and plasminogen deficient mice using the PymT mouse mammary tumour

model.^{100 101} Although angiogenic endothelial cells require uPA and plasmin to degrade extracellular matrix components and to migrate, plasmin proteolysis needs to be controlled by a physiological inhibitor, such as PAI-1, to allow the stabilisation of the surrounding matrix and the assembly of endothelial cells into channels.^{2 102} The importance of balanced proteolysis in the process of angiogenesis might provide an explanation for the dual role of proteinases and inhibitors in angiogenesis. Indeed, surprisingly, PAI-1 is a strong negative prognostic marker in different types of cancer.^{12 103} This could be explained by a simultaneous enhancement in uPA and PAI-1 expression, resulting in a net excess of proteolytic activity. Alternatively, this paradoxical clinical observation may be related to a potential direct role of PAI-1 in cancer cell migration and invasion. Studies in PAI-1 deficient mice have revealed that PAI-1 is essential for the vascularisation of skin tumours.^{94 96} The requirement for host PAI-1 during tumour angiogenesis has been confirmed in a fibrosarcoma model⁹⁷ and in the mouse aortic ring model.⁹⁵ However, it is likely that PAI-1 is not of general importance in tumour progression and its angiogenic effect might be dependent on the tumour type, its cellular source (tumour cells versus host cells) (personal unpublished data, 2003), its concentration, and the step of cancer progression that is being considered. Indeed, PAI-1 deficiency did not affect sarcoma development,⁹⁸ melanoma cell metastasis,¹⁰⁴ or primary tumour growth and dissemination in a genetic model of mammary adenocarcinoma.¹⁰⁵ Recently, a dose dependent effect of PAI-1 has been demonstrated in the aortic ring assay,⁹⁵ in bFGF induced angiogenesis,¹⁰⁶ and in choroidal angiogenesis.¹⁰⁷ Therefore, PAI-1 appears to be proangiogenic at physiological concentrations and antiangiogenic at high, pharmacological concentrations.

"Surprisingly, plasminogen activator inhibitor 1 is a strong negative prognostic marker in different types of cancer"

In addition to its ability to control pericellular proteolysis, PAI-1 could act through its capacity to interact with uPAR, integrins, and vitronectin, thereby controlling cell migration.^{12 108-110} Therefore, PAI-1 can competitively inhibit the uPAR dependent attachment of cells to vitronectin. Recently, PAI-1 has been shown to detach cells by disrupting uPAR-vitronectin and integrin-vitronectin interactions.¹¹¹ To investigate the mechanism of action of PAI-1, adenoviruses were used to deliver two mutant forms of PAI-1 to PAI-1 deficient mice in the transplantation system of malignant keratino-cytes⁹⁶ and in the aortic ring assay.⁹⁵ In both models, angiogenesis is restored only by the PAI-1 mutant that retains plasminogen activator inhibitory activity, but not by the mutant that can interact with vitronectin but has impaired proteolytic control. These findings demonstrate that, in these models, PAI-1 requirement is merely related to its capacity to inhibit excessive proteolysis, rather than to interact with vitronectin. In a separate study using PAI-1 mutants, the inhibition of bFGF induced angiogenesis in the CAM assay requires both the antiprotease activity of PAI-1 and its vitronectin binding capacity.¹⁰⁶ Therefore, the mechanism of PAI-1 action will probably be dependent on the experimental setting, the in vivo situation, and the type of cancer.

Role of MT1-MMP during tumoral angiogenesis and paradoxical functions of TIMPs

The recent generation of MT1-MMP deficient mice revealed its involvement in skeletal development.^{91 112} The role of MT1-MMP during tumour angiogenesis has only been recently established. The overexpression of MT1-MMP in human melanoma,¹¹³ breast adenocarcinoma,¹¹⁴ and glioma cells^{115 116} is associated with enhanced in vitro invasion and increased in vivo tumour growth and vascularisation. MT1-MMP might contribute to tumour angiogenesis through different mechanisms including at least: (a) the activation of $\alpha v \beta 3$ integrin, which plays a major role during angiogenesis¹¹⁷; (b) fibrinolytic activity^{44 45}; (c) collagenolytic activity¹¹⁸; (d) pericellular proteolysis, directly or indirectly, by activating pro-MMP2 and pro-MMP-13 and thereby promoting cell invasion and migration; and (e) the transcriptional regulation of VEGF expression.^{114 116}

Consistent with the prevailing notion that angiogenesis requires at least MT-MMP mediated proteolysis, TIMPs have been shown to suppress not only tumour invasion and metastasis, but also tumour growth and neovascularisation in several tumour models.^{29 119-123} Paradoxically, the association between poor prognosis and high levels of expression of TIMPs has been reported for several cancer types.^{124 125} An increasing body of evidence suggests that TIMPs are multifunctional proteins that can have both an inhibitory or stimulatory effect on tumorigenesis.²⁹ TIMPs promote the proliferation of some cell types, and their antiapoptotic effects may favour tumour expansion during the onset and early growth of the primary tumour.^{29 126-128} The antiangiogenic activity of TIMP-2 could be linked to the direct control of MMP proteolysis, an inhibitory effect on endothelial cell proliferation,¹²⁹ and/or the downregulation of VEGF.¹³⁰ In contrast, TIMP-2 also plays a role in the activation of pro-MMP-2 as an adaptor molecule.^{36 54} A ternary complex composed of MT1-MMP-TIMP-2-pro-MMP-2 is thought to cluster pro-MMP-2 at the cell surface near a TIMP-2 free MT1-MMP molecule, initiating the processing of the bound pro-MMP-2 (fig 4). This process could be facilitated by MT1-MMP oligomerisation.^{131 132} According to this model, the activation of pro-MMP-2 at the cell surface is regulated by

the balance between the MT1-MMP-TIMP-2 complex (MMP-2 receptor) and TIMP-2 free MT1-MMP (pro-MMP-2 activator). Therefore, like PAI-1, the effect of TIMP-2 on angiogenesis could be dose dependent. In addition, the expression of MT1-MMP by tumour cells has been shown to mediate the internalisation and intracellular degradation of TIMP-2, thereby altering the balance between active MMPs and TIMPs.⁵³

CONCLUDING REMARKS

The establishment of a causal relation between protease expression and tumour progression has prompted the development of MMP inhibitors, uPA or uPAR antagonists, inhibitors of PAI-1, and MMP inhibitors combined with the N-terminal fragment of uPA.^{10 12 27 87 133 134} Because studies have indicated a functional overlap between SPs and MMPs in wound healing,^{133 136} a similar functional overlap is expected in cancer invasion. Therefore, arresting different steps of cancer progression will require the combined use of inhibitors of both classes. Undoubtedly, future studies of recently discovered or new membrane associated proteases will be crucial to the better understanding of the role of pericellular proteolysis in cancer and for the development of new anticancer strategies.

Take home messages

- Serine proteases (plasminogen-plasmin system) and matrix metalloproteases (MMPs) are overexpressed in many human cancers
- The activity of serine proteases is controlled by physiological inhibitors, namely: plasminogen activator inhibitors (PAI-1 and PAI-2) and tissue inhibitors of metalloproteases (TIMPs)
- Their contribution to cancer invasion and metastasis formation was initially related to their capacity to degrade extracellular matrix components, although they now appear to be multifunctional molecules, whose list of substrates has recently been extended to at least cell surface molecules, growth factors, growth factor binding proteins, and cytokines/chemokines
- New membrane associated proteases have been identified: a disintegrin and metalloproteases, membrane-type MMPs, and type II serine proteases. Together with the plasminogen-plasmin system they control cell surface proteolysis, which has emerged as an important mechanism to regulate cell growth, migration, invasion, metastasis, and angiogenesis
- Dual functions of their physiological inhibitors have been identified, explaining why high rather than low concentrations of PAI-1 and TIMPs predict poor survival in human cancer

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