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No grip, no growth: the conceptual basis of excessive proteolysis in the treatment of cancer

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Abstract

The formation of new bloodvessels, called angiogenesis, is critical for a tumour to grow beyond a few mm^3 in size. A provisional matrix promotes endothelial cell adhesion, migration, proliferation and survival. Synthesis and degradation of this matrix closely resemble processes that occur during coagulation and fibrinolysis. Degradation of the matrix and fibrinolysis are tightly controlled and balanced by stimulators and inhibitors of the plasminogen activation system. Here we give an overview of these processes during tumour progression. We postulate a novel way to inhibit angiogenesis by removal of the matrix through specific and localised overstimulation of the plasminogen activation system.

Keywords: Angiogenesis; Therapeutic strategies; Metastasis; Extracellular matrix; Plasminogen; Fibrin; TAFI; Endostatin; Angiostatin

1. Introduction

Preclinical and clinical data have demonstrated that angiogenesis, the formation of new blood vessels from pre-existing ones, is essential for the growth of tumours and their metastasis. Therefore, the discovery or development of molecules that inhibit angiogenesis may lead to a better treatment of cancer [1].

Since the late 19th century, abnormalities in the haemostatic system have frequently been reported in cancer patients, including thromboembolic and bleeding disorders [2]. The recent finding that a number of potent naturally occuring inhibitors of angiogenesis, such as anti-angiogenic anti-thrombin III [3] and angiostatin [4], are derived from proteins that play a role in haemostasis has strengthened the idea that the haemostatic system plays a crucial role in angiogenesis and tumour growth [5]. It has become apparent that coagulation and fibrinolysis support the formation and degradation of a provisional matrix, which facilitates angiogenesis. The critical role of the coagulation and fibrinolytic systems make them excellent tools for antiangiogenic and antitumorigenic therapy. Here we will review the role of the fibrinolytic system in angiogenesis and tumour growth. We discuss components of this system, evaluate its inhibitors for use in antiangiogenic therapy and provide a novel hypothesis for inhibiting angiogenesis.

2. Angiogenesis and the formation of a provisional matrix

The formation of a provisional extracellular matrix is a hallmark of angiogenesis. This occurs after vascular injury, during inflammation, and in tumours [6,7]. Angiogenic factors, most notably vascular endothelial growth factor (VEGF), that are produced by the tumour induce hyperpermeability resulting in the extravasation of plasma proteins, including fibrinogen, prothrombin, vitronectin and many others. In addition, angiogenic factors, including VEGF, induce expression of tissue factor on the endothelial cells [8]. Tissue factor, which is not only present on stimulated endothelial cells, but also in the subendothelial matrix and on many tumour cells [9,10] triggers the formation of fibrin. Exposure of tissue factor leads to thrombin activation, and as a result fibrin is formed by polymerisation of thrombin-cleaved fibrinogen. Together with other adhesive proteins, such as vitronectin, laminin and

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fibronectin, fibrin forms the provisional matrix. The provisional matrix supports tissue remodelling, wound healing, angiogenesis and tumour growth (reviewed in [11]). Fibrin and the other components of the extracellular matrix are involved in the regulation of cell proliferation, migration and survival or apoptosis through interactions with adhesion molecules on the cell surface. Important adhesion molecules include the receptors for fibrin and vitronectin, the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, (reviewed in [12]). These interactions of the endothelial cells with the provisional matrix are crucial. Vitronectin can protect endothelial cells from apoptosis [13], while antibodies against its receptor $\alpha_v\beta_3$ induce apoptosis [14,15]. Through interactions with plasminogen activator inhibitor 1 (PAI-1) and other components of the plasminogen activation system vitronectin is also an important regulator of plasmin formation and thereby controls the proteolysis of the provisional matrix. During angiogenesis the provisional matrix is continuously remodelled by balanced degradation and resynthesis (Fig. 1). The generation and subsequent breakdown closely resemble the processes of coagulation and fibrinolysis.

3. The plasminogen activation system

The plasminogen activation system, which leads to the formation of the serine protease plasmin and subsequent fibrinolysis, has been shown to play an important role in the breakdown of the provisional matrix. Angiogenic growth factors induce the expression of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) on the surface of endothelial cells [16,17]. Both tPA and uPA are serine proteases that can generate plasmin by proteolytic cleavage of its zymogen plasminogen. Plasminogen, like fibrinogen and other plasma components of the provisional matrix, is synthesised in the liver and deposited in response to hyperpermeability. The formation of plasmin is essential for the invasion and migration of endothelial cells into the tissue to be vascularised. The plasminogen activation system is not limited to endothelial cells. While tPA is almost exclusively expressed by endothelial cells [18], uPA also facilitates migration of other cells like epithelial cells, fibroblasts and tumour cells [19,20]. A variety of cell types can bind components of the fibrinolytic system, including plasminogen [21], plasmin [22], uPA [[22] and tPA [24]. Annexin II, a cellular receptor of tPA, enhances tPA activity more than 50-fold [25]. Plasminogen concentration is increased on the cell surface by binding to α-enolase [26,27]. Besides fibrin, plasminogen can bind a variety of extracellular matrix proteins, including laminin, fibronectin, vitronectin and collagen [28–30]. Plasmin causes proteolysis of the extracellular matrix by degrading fibrin into fibrin degradation products (FDP), called fibrinolysis, and other matrix proteins directly. In addition, plasmin can activate several metalloproteinases (MMPs) which further degrade the extracellular matrix. Activation of

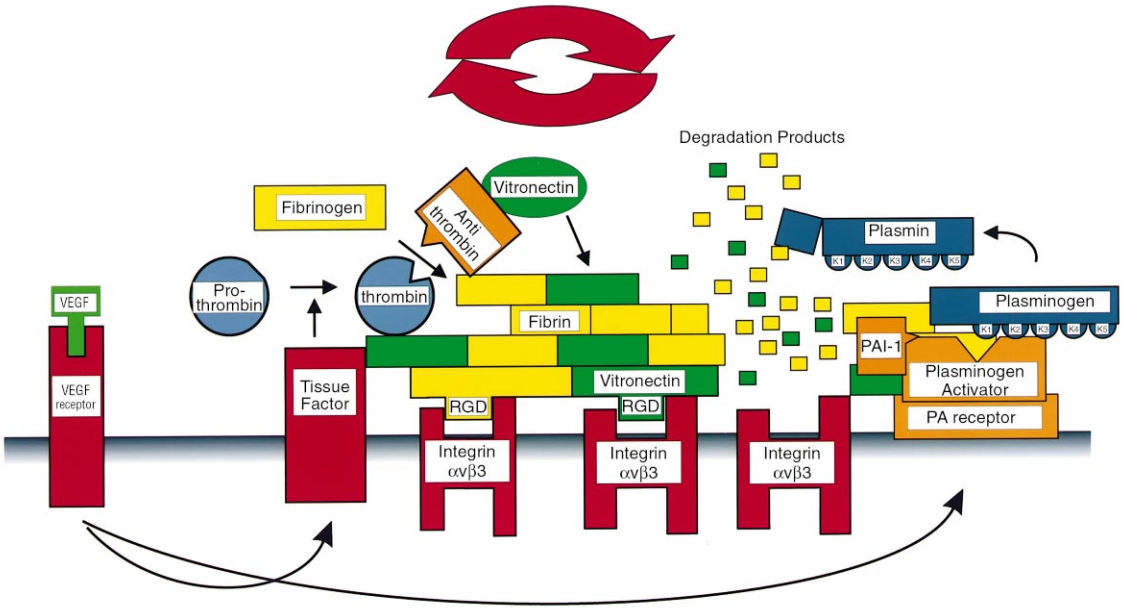

Fig. 1. Coagulation and fibrinolysis on the cell surface. Upon stimulation by vascular endothelial growth factor (VEGF), components that initiate and control coagulation and fibrinolysis are upregulated. The continuous formation and breakdown of the provisional matrix is of great importance for cell viability, growth and motility. Matrix components support adhesion of endothelial cells and degradation of the matrix is necessary for migration. RGD, amino acids involved in binding of extracellular matrix proteins by integrins; PAI, plasminogen activator inhibitor; $\alpha_v\beta_3$, integrin receptor for vitronectin and fibrin(ogen).

plasminogen is tightly controlled by several protease inhibitors, plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2) and α2-antiplasmin [31,32]. Plasmin, if not bound to fibrin or the cell surface, is rapidly inhibited by α2-antiplasmin [33].

4. Lysine residues and lysine binding sites

Interactions of plasminogen with its receptors and extracellular matrix proteins are mediated by five kringle domains that are present in plasminogen. These kringle domains contain high affinity binding sites for lysine residues, especially when these residues are located at the carboxy-terminus of proteins (see below). Plasmin always cleaves after a lysine residue and thereby generates a free carboxy-terminal lysine residue. In the case of fibrin, free carboxy-terminal lysine residues bind new plasminogen molecules and tPA with high affinity resulting in an increased rate of plasminogen activation [34,35]. In contrast to uPA, the activity of tPA depends on the presence of such cleaved fibrin fragments (FDP). Thus, partially degraded fibrin, containing carboxy-terminal lysine residues serves as cofactor in the enhanced formation of plasmin (Fig. 2). The carboxy-terminal lysine residues are of critical importance since their removal by carboxypeptidase B type enzymes can completely abrogate the cofactor function [36–38].

5. Thrombin-activatable fibrinolysis inhibitor (TAFI)

TAFI, also known as plasma procarboxypeptidase B, procarboxypeptidase U or procarboxypeptidase R, is a recently identified regulator of the plasminogen activation system (reviewed in [39]). Like plasminogen and fibrinogen, TAFI is made by the liver. TAFI is activated following coagulation and cleaves carboxy-terminal lysine and arginine residues from plasmin degraded fibrin [40]. This prolongs the clot lysis time due to a decrease in the rate of plasminogen activation [41,42]. TAFI can be activated *in vitro* by high concentrations of trypsin [43], thrombin [41] or plasmin [44]. Activation by plasmin can be improved by heparin [45]. Most importantly, activation of TAFI by thrombin is increased 1250-fold in the presence of thrombomodulin [46,47], a receptor almost exclusively expressed on endothelial cells. Since thrombomodulin expression can be upregulated by VEGF [48] it is likely that the activity of TAFI is regulated during angiogenesis. Recently, *in vivo* studies revealed that inhibition of TAFI by potato carboxypeptidase inhibitor can enhance tPA-induced thrombolysis [49]. Taken together, TAFI is expected to control plasmin-mediated proteolysis of the provisional matrix during angiogenesis.

6. Antifibrinolytic therapy and cancer

Given plasmin's pivotal role in angiogenesis and tumour growth, drugs that target the formation of plasmin are expected to affect angiogenesis and cancer progression. Indeed, results from many studies have revealed promising antiangiogenic and antitumorigenic activity of inhibitors that affect plasmin formation. Several agents that inhibit fibrinolysis either by interfering with plasminogen activation or plasmin activity have been tested both *in vivo* and *in vitro* (Table 1).

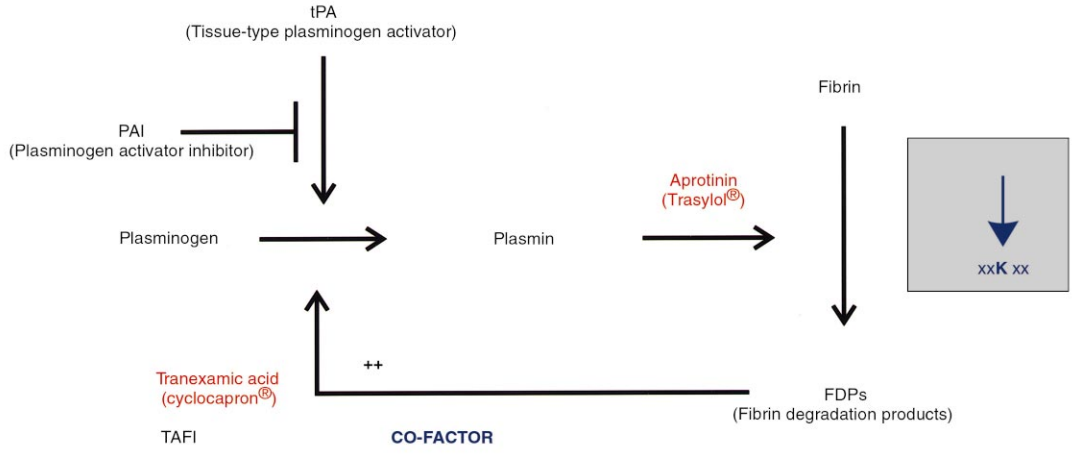

Fig. 2. The tissue-type plasminogen (tPA)-mediated plasminogen activation system. The inactive zymogen plasminogen can be activated into the serine protease plasmin by tPA which in turn degrades fibrin. The activity of tPA is greatly enhanced by fibrin degradation products (FDP) which are obtained after plasmin cleavage of fibrin. The stimulatory activity of FDP is critically dependent on the presence of carboxy-terminal lysine residues. Thrombin-activatable fibrinolysis inhibitor (TAFI) can block the activity of FDP by removing the carboxy-terminal lysines. Aprotinin (Trasylol®) and tranexamic acid (Cyclokapron®) inhibit fibrinolysis by blockage of the plasmin activity and co-factor function of FDP, respectively.

Table 1
Antiangiogenic and antitumorigenic compounds that deregulate the plasminogen activation system[a]

Compound/Mechanism	Effect *in vivo*/*in vitro* [Ref.]
N-terminal fragment of uPA fused to IgG, uPA antagonist	Arrests metastasis, inhibits establishment of primary tumours and micrometastases [56]
N-terminal fragment of uPA fused to IgG (m1-48Ig), uPA antagonist	*In vivo* suppression of basic fibroblast growth factor-induced neovascularisation and B16 melanoma growth in syngenic mice [51]
N-terminal fragment of uPA ligated to HSA (ATF-HSA), uPA antagonist	*In vitro* inhibition of tumour cell invasion in matrigel, changes in cell morphology and remodelling of cytoskeleton [52,53]
Soluble uPAR	*In vitro* inhibition of human microvascular endothelial cells capillary formation in Matrigel [54]
PAI-1 extended half-life, uPA inhibitor	Inhibits prostate cancer xenografts [55]
p-Aminobenzamidine	Inhibits growth of a human prostate tumour in mice and migration of endothelial cells in Matrigel [55,56]
Amiloride	Inhibits growth of a human prostate tumour in SCID mice [55]
Anticatalytic uPA	Inhibits the formation of lung metastases of Lewis lung carcinoma and suppresses invasion of tumour cells through Matrigel [57]
Anticatalytic (human) uPA	No invasion of human carcinoma HEp3, however, no reduced incidence of distant metastases in mice [59]
Anticatalytic (human) uPA	No inhibition of human carcinoma HEp3 at the site of primary inoculation on the chorioallantoic membrane, but prevention of metastasis to the embryo lung [59]
Inactive recombinant murine u-PA that retains receptor binding	Inhibits prostate cancer neovascularisation, metastasis and growth in rat [60]
Aprotinin (Trasylol®), inhibitor of plasmin and other serine proteases	Inhibits invasion of endothelial cells on the human amniotic membrane [61], tube formation in matrix gels [54,62] and metastasis of Lewis lung carcinoma in mice [63]
Tranexamic acid (Cyclokapron®) and epsilon-amino-caproic acid (ACA), lysine-analogue which inhibits binding of plasminogen to its substrates	Inhibits invasion and migration of endothelial cells on the human amniotic membrane [61], antiangiogenic in the cornea assay in rabbits [64] and *in vitro* angiogenesis [54]. Inhibits growth of V2 carcinoma in rabbits [65], of Lewis lung carcinoma in mice [66], of (human) tumours in mice [67–72]. Beneficial effects in humans have been reported [63,71–79]
α2-Antiplasmin, inhibitor of plasmin	Inhibits *in vitro* tumour cell invasion through the human amniotic membrane [61]
Angiostatin and other fragments of plasmin, unknown mode of action (see text)	Inhibits proliferation and migration of endothelial cells, neovascularisation in the corneal assay and subcutanous tumours [80–85]
Endostatin, unknown mode of action (see text)	Inhibits primary tumour growth and metastasis [86–88], angiogenesis in CAM [89] and endothelial cell proliferation and migration [90], induces endothelial cell apoptosis [91]
Streptokinase and tPA, plasminogen activators which increase fibrinolysis	Inhibits pulmonary tumour seeding in an animal model [92,93]
Potato carboxypeptidase inhibitor (PCI), inhibitor of TAFI, enhances fibrinolysis [49]	Inhibits the growth of several human pancreatic adenocarcinoma cell lines in nude mice [94]

[a] For optimal angiogenesis to occur, plasmin formation and action needs to be under stringent control of activators, including tPA, and inhibitors, such as PAI-1 and α2-antiplasmin. A shift in the balance, by either increasing the levels or activity of inhibitors or by enhancing the formation of plasmin has been shown to have profound effects on either endothelial cell adhesion, migration, angiogenesis, metastasis or tumour growth.

SCID, severe combined immunodeficiency; HSA, human serum albumin; CAM, chicken choriallantoic membrane.

7. Tranexamic acid

Tranexamic acid (Cyclokapron®) is a lysine analogue that blocks the interaction between lysine residues and the lysine binding sites that are present in the kringle domains of plasminogen. Several preclinical as well as clinical studies have reported promising effects of this drug on cancer growth. Tranexamic acid was shown to inhibit the growth of human lung, ovarian and renal carcinomas transplanted into nude mice. Inhibition was apparently caused by increased fibrin depositions at the advancing border of tumours due to reduced fibrinolytic activity [67]. Profound effects of tranexamic acid were seen on the growth of lung, breast, hepatoma and ovarian carcinomas in other mice models [68,69]. In a study by Tanaka and colleagues, remarkable effects (60%) on the occurrence of metastases of Lewis lung carcinoma in mice were seen when mice were treated with 500 mg/kg twice daily [66]. In human mammary carcinoma and melanoma cells, tranexamic acid inhib-

ited the binding of plasmin and plasminogen to the cell surface [95]. Another lysine analogue, epsilon-aminocaproic acid (εACA), decreased the tPA-mediated fibrinolytic activity of Co115 colon carcinoma cells *in vitro* [96]. Tumour-induced corneal angiogenesis could be significantly reduced by tranexamic acid and εACA [64]. Furthermore, εACA has been shown to inhibit glioma tumour growth in a mouse model [70] and *in vitro* angiogenesis [54]. Stabilisation of active TAFI by εACA, determined by using a small substrate for TAFI, may give an additional inhibitory effect on fibrinolysis and angiogenesis [43,97]. A few promising clinical studies have been carried out to test the effect of tranexamic acid in humans. Patients with ovarian cancer showed stable disease with a median survival of 12.5 months after treatment with 4–6 g/day tranexamic acid [72]. In another study, 6 out of 11 stage II or IV ovarian cancer patients responded to tranexamic acid therapy after surgical tumour debulking [73]. Taken together, since tranexamic acid has profound effects on angiogenesis and tumour growth and has no serious side-effect, it is a potential candidate angiogenesis inhibitor. Better results might be obtained by continuous treatment with tranexamic acid. In contrast to daily administration, continuous delivery of angiogenesis inhibitors has been shown to be much more effective [98].

8. Inhibitors of uPA

Inhibitors of uPA that either affect uPA activity or prevent uPA binding to its receptor have been succesfully applied *in vitro* and *in vivo*. Antibodies against uPA block tumour metastases in the chorioallantoic membrane assay [59]. Furthermore, antibodies against uPA were used to inhibit metastasis and tumour growth in mice models [57–99]. Growth and formation of metastases of human cancer cell lines were inhibited after treatment with an uPA antagonist [50]. Non-catalytic uPA was coupled to IgG and tested *in vivo*. In an experimental metastases model, treatment with this fusion protein resulted in a decreased number of micrometastases in the lung ranging between 5 and 30% of vehicle-treated mice. This demonstrates that competitive inhibition of uPA can arrest metastasis and primary tumour growth. Furthermore, establishment of primary tumours was abrogated since a single dose of uPA-IgG administered 1 h prior to tail vein injection of the cells reduced lung colony formation to just 3.5% of vehicle-treated severe combined immunodeficiency mice. Min and associates obtained comparable results using a similar approach [51]. Ligation of the epidermal growth factor (EGF) domain of uPA to IgG resulted in a potent antagonist of uPA which inhibited capillary tube formation, basic fibroblast growth factor (bFGF) induced neovascularisation and B16 melanoma growth in syngenic mice. A similar construct was made by Lu and associates, who fused the amino-terminal fragment of uPA to human serum albumin. This construct inhibited *in vitro* tumour cell invasion and endothelial cell mobility and deformability [52,53]. Inactive uPA, generated by PCR mutagenesis, that retains receptor binding reduced prostate cancer neovascularisation and growth [60]. Prevention of *in vitro* tube formation in three-dimensional fibrin matrices was achieved with soluble uPAR and antibodies that inhibit uPA activity [54]. Others have used a physiological inhibitor of plasminogen activation, PAI-1, whose half-life was extended by mutation [55]. Synthetic inhibitors of uPA activity like p-aminobenzamidine and amiloride showed a clear decrease in tumour-growth rate compared with untreated mice [55,56]. These results demonstrate that blockage of uPA by uPA inhibitors can reduce tumour size in experimental animals. Agents, such as aprotinin, which inhibit plasmin activity directly, can also inhibit metastasis [63].

9. Angiostatin

The observation that in some cases removal of a primary tumour in patients may lead to the rapid growth of previously undetected metastases [100,101] suggests that primary tumours make factors that may inhibit the outgrowth of distant tumours [102]. Based on this concept, the angiogenesis inhibitor angiostatin was found [4]. Angiostatin is a proteolytic fragment of plasmin(ogen) and consists of the kringle domains 1–4 of plasminogen. The molecule was purified from urine of tumour-bearing mice using lysine affinity chromatography. Systemic administration of angiostatin blocked neovascularisation and growth of metastasis in the absence of the primary tumour. At higher doses, angiostatin can inhibit growth of primary tumours as well [80,81]. Continuous delivery of angiostatin has been shown to be more effective in inhibiting angiogenesis than bolus injections [98]. *In vitro*, angiostatin inhibits bFGF-induced endothelial cell proliferation and migration [4,103]. *In vivo* generation from plasmin(ogen) has been demonstrated and can be achieved by several proteases [104–108]. Angiostatin binds to the α/β-subunits of adenosine triphosphate (ATP) synthase on the surface of endothelial cells causing cytolysis [109]. Presently, the biological relevance of these findings is unclear. It is not unlikely that angiostatin exerts its effect by binding integrin ligands present in the extracellular matrix [110] thereby inducing changes in intracellular signalling [111]. Alternatively, angiostatin may effect plasmin-mediated proteolysis through non-competitive inhibition of tPA activity [112]. Also, profibrinolytic effects have been described for kringle domains 1–3, which can block the interaction of plasmin

with α2-antiplasmin [113]. Finally, angiostatin binds tissue factor and may regulate the formation of a provisional matrix through an effect on coagulation [114]. In conclusion, although direct effects on endothelial cells are described, angiostatin might be involved in the generation and breakdown of the provisional matrix as well.

10. Endostatin

In 1997 a carboxy-terminal fragment of collagen XVIII, named endostatin, was identified that proved to be a potent inhibitor of endothelial cell proliferation and angiogenesis [90]. Endostatin was purified from conditioned media of haemangioendothelioma (EOMA) cells using a heparin affinity column. When administered to mice bearing Lewis lung carcinoma, T241 fibrosarcoma or B16F10 melanoma, recombinant mouse endostatin caused tumour regression without developing drug resistance [86]. Endostatin treatment of cow pulmonary artery endothelial cells caused apoptosis [91]. Like plasminogen and fibrinogen, collagen XVIII is made by hepatocytes [115]. Recombinant mouse endostatin produced by mammalian cells was shown to bind to heparin with a K(d) of 0.3 μM, suggesting that this interaction may play a role in its antiangiogenic activity. Mutations in endostatin that affect heparin binding abolished endostatin-mediated inhibition of bFGF-induced angiogenesis in a chick chorioallantoic membrane assay [89]. However, binding of endostatin to blood vessels was independent of heparan sulphate and endostatin did not compete with bFGF [116]. It has been shown that EOMA cells produce elastase which can process collagen XVIII to endostatin [117]. We have recently found a powerful molecular activity for endostatin that provides an explanation as to how endostatin may act as an antitumorigenic compound (data not shown). Because endostatin is a fragment of an extracellular matrix component, exerts its effects via the tumour vasculature and has a carboxy-terminal lysine, we examined whether endostatin can regulate plasmin formation. We found in a subcutaneous colon carcinoma model that the antitumour activity of endostatin was completely abolished when mice were also treated with carboxypeptidase B. This suggests an important role for the carboxy-terminal lysines for the antitumour activity of endostatin. The finding that endostatin purified from plasma of cancer patients lacks the carboxy-terminal lysine and is inactive in inhibiting endothelial cell proliferation [118] is in agreement with our findings. We established that endostatin binds plasminogen and stimulated tPA-mediated plasmin formation in a lysine-dependent manner. As has also been shown for fibrin, binding of endostatin to tPA could not be inhibited by carboxypeptidase B or lysine analogues. Our results point to a novel mechanism in which overstimulation of the plasminogen system may inhibit angiogenesis and tumour growth (see below).

11. Can hyperfibrinolysis inhibit angiogenesis?

Currently, a well accepted model to explain angiogenesis and the angiogenic switch is based on a balance between stimulators and inhibitors. Depending on the levels of stimulators and inhibitors a tumour will grow or remain dormant [4,102,119]. uPA and tPA are currently referred to as pro-angiogenic, whereas PAI is called an inhibitor of angiogenesis [5,120–123]. It has been suggested that angiogenic factors which induce angiogenesis induce endothelial expression of both uPA and PAI-1, with a slight excess in favour of the protease [124]. However, paradoxically PAI-1 has been correlated with a poor prognosis for many cancers (reviewed in [125]). Furthermore, in PAI-1 knockout mice, invasion of malignant keratinocytes and angiogenesis was abrogated, which could be restored by a PAI-1-expressing adenoviral vector [126]. This shows an important and essential pro-angiogenic role for PAI-1. Another example is the inhibitor thrombospondin, which was found to be essential for pathological angiogenesis during wound healing in knockout mice [127]. We state that these 'negative' regulators of angiogenesis are indispensable and stabilise this balanced process by limiting excessive proteolysis (see below). We propose an haemostasis model for angiogenesis. Our model considers angiogenesis depending on a perfect balance of coagulation and fibrinolysis (Fig. 3). Disturbance of this balance by inhibition, but also by overstimulation of fibrinolysis might prevent angiogenesis. This implies that widely accepted pro-angiogenic factors like uPA, tPA and plasmin can be antiangiogenic as well when administered at higher doses.

The concept of plasmin-mediated inhibition of tumour growth is supported by our recent finding that continuous systemic treatment of mice with tPA, which efficiently generates plasmin *in vivo* and is used clinically in patients with myocardial infarction, also inhibits tumour growth (data not shown). Moreover, others have demonstrated *in vitro* that induction of plasminogen activation leads to endothelial cell detachment [130], inhibition of cell adhesion [131] or endothelial cell destruction [132]. Enhanced formation of plasmin, through administration of tPA or streptokinase (another plasminogen activator), also reduced pulmonary tumour seeding in an experimental animal model [92,93]. Moreover, maspin, another stimulator of tPA, inhibits angiogenesis [143,144]. Our model may explain the observation that in the absence of PAI, which may lead to increased plasminogen activator activity and plasmin formation, vascularisation and tumour invasion is prevented [126]. Additionally, patients with

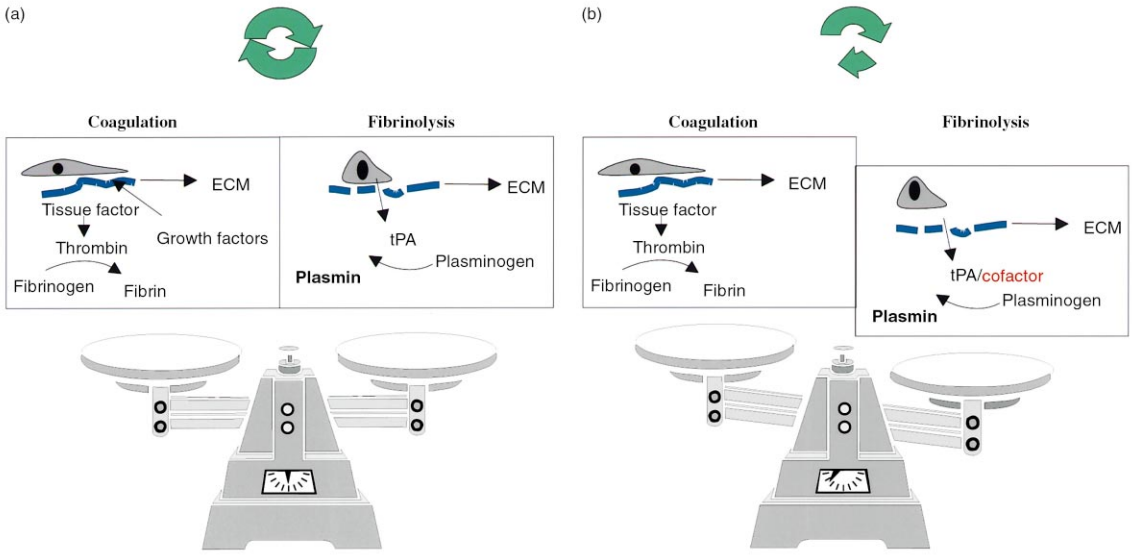

Fig. 3. Angiogenesis is a continuous balanced process. Many tumours contain fibrin depositions [128,129] and elevated levels of plasminogen activators and PAI-1 (reviewed in [125]). In cancer patients levels of fibrinogen degradation products, as well as complexes between plasmin and α_2-antiplasmin, are elevated. This indicates an increased turnover of fibrinogen and plasminogen. During angiogenesis the provisional matrix is continuously degraded and resynthesised, called remodelling. This process is similar to coagulation and fibrinolysis. Generation of a temporary matrix is induced by tissue factor (coagulation) which is followed by degradation through plasmin (fibrinolysis) (left panel). The formation of fibrin polymers and release of fibrin degradation products after plasmin-mediated proteolysis is a highly regulated and balanced process required for endothelial cell growth and angiogenesis. Overstimulation of tissue-type plasminogen activator which leads to excess plasmin formation and hyperfibrinolysis can disturb this balance and prevent angiogenesis (right panel).

peripheral tumours that are reduced by tumour necrosis factor alpha (TNFα) show elevated concentrations of tPA [133–136] and increased levels of plasmin-α2 antiplasmin (PAP) [135] and fibrin degradation products (FDP) [134,137]. Increased levels of PAP and FDP strongly suggest active fibrinolysis that correlates with tumour reduction. There might also be an additive effect of plasminogen activator activity on the migration of macrophages [138]. Inhibition of macrophage mobility will deprive tumour cells from growth factors. Similarly, excessive fibrinolysis might affect platelet interactions with fibrin in the tumour vasculature, thereby inhibiting angiogenesis [139]. Taken together we propose that molecules that lead to excessive proteolysis in the tumour may be powerful antiangiogenic and antitumorigenic agents.

12. Cryptic fragments

An increasing number of proteolytic fragments, some of which may be generated naturally, have been described with potent antiangiogenic activity. These include angiostatin [4], endostatin [92], antiangiogenic antithrombin III [3], restin [140], canstatin [141], kringle domain 5 of plasminogen [82] and thrombin fragment 1 and 2 [142]. We recently found that fragments of fibrin (FDP) also possess antitumorigenic activity, possibly via a tPA-mediated mechanism similar to endostatin (data not shown). At present it is unclear why these fragments are generated by tumours, whether they have a normal physiological role, and whether these fragments are generated during other (patho)-physiological processes in which angiogenesis is involved. Because FDP have an important regulatory role in the control of fibrinolysis it may be that in analogy, other endogenous 'cryptic' fragments serve a similar and normal physiological role, regulating tissue remodelling by controlling coagulation or fibrinolysis. These factors may only act antiangiogenically because they are administered at doses that are in excess of endogenous levels, thereby disturbing strictly balanced proteolysis.

13. Concluding remarks

The interaction of endothelial cells and the extracellular matrix forms an important area of investigation. Based on the concept that degradation of the extracellular matrix is a critical step in the progression of cancer, therapeutic strategies have been developed to prevent this. Inhibitors of metalloproteinases have shown biological activity in preclinical models and are currently being tested in phase III clinical trials. A significant limitation of this approach is that the use of proteolytic inhibitors will never lead to the removal of tumour stroma and therefore of the tumour itself. In fact, ultimately, the tumour and its stroma need to be

removed by proteolysis. We would argue that drugs that enhance proteolysis may give far better results and may induce tumour regression. In this review, we discussed whether excessive localised proteolysis may be achieved by specifically activating tPA at the sites of angiogenesis. The feasibility of this approach has been demonstrated in preclinical models. The conceptual basis of localised excessive proteolysis ('no grip, no growth') will facilitate the development of an array of compounds that may be used in angiogenesis-related diseases.

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