Plasminogen activation in cancer

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Chapter 1

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

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Summary

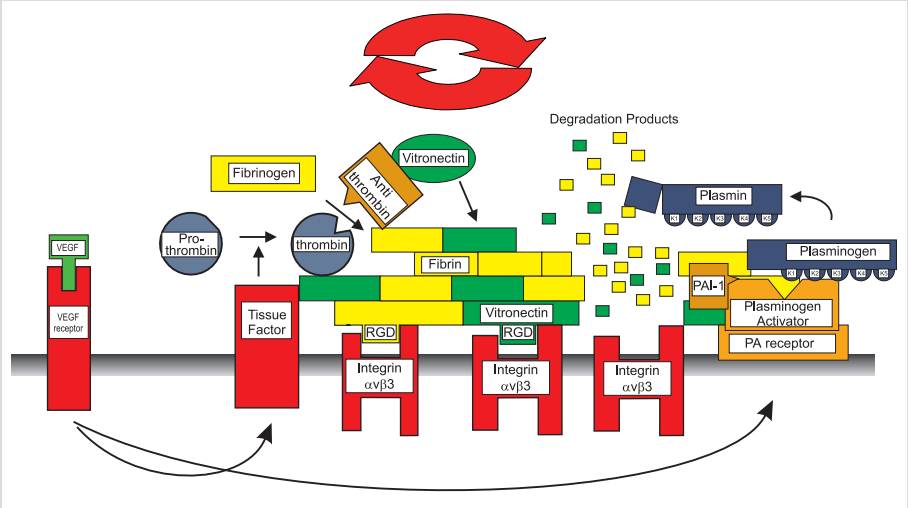
The formation of new blood vessels, called angiogenesis, is critical for a tumor to grow beyond a few mm^3 in size. During angiogenesis, a provisional matrix promotes endothelial cell adhesion, migration, proliferation and survival. Synthesis and degradation of this matrix closely resemble processes 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 tumor progression. Furthermore, we postulate a novel way to inhibit angiogenesis by removal of the matrix through specific and localized overstimulation of the plasminogen activation system.

Preclinical and clinical data have demonstrated that angiogenesis, the formation of new blood vessels from pre-existing ones, is essential for the growth of tumors 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 hemostatic system have frequently been reported in cancer patients, including thromboembolic and bleeding disorders[2]. The recent finding that a number of potent naturally occurring inhibitors of angiogenesis, such as antiangiogenic anti-thrombin III[3] and angiostatin[4], are derived from proteins that play a role in hemostasis has strengthen the idea that the hemostatic system plays a crucial role in angiogenesis and tumor 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 system makes them excellent tools for antiangiogenic and antitumorigenic therapy. Here we will review the role of the fibrinolytic system in angiogenesis and tumor growth. We discuss components of this system, evaluate its inhibitors for use in antiangiogenic therapy and provide a novel hypothesis for inhibiting angiogenesis.

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 tumors[6;7]. Angiogenic factors that are produced by the tumor, most notably vascular endothelial growth factor (VEGF), 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 tumor cells triggers the formation of fibrin[9;10]. Exposure of tissue factor leads to thrombin activation, and as a result fibrin is formed by polymerization of thrombin cleaved fibrinogen. Together with other adhesive proteins, such as vitronectin, laminin and fibronectin, fibrin forms the provisional matrix. The provisional matrix supports tissue remodeling, wound healing, angiogenesis and tumor 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 remodeled by balanced degradation and resynthesis (Figure 1).

Figure 1.



Coagulation and fibrinolysis on the cell surface. Upon stimulation by 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).

The generation and subsequent breakdown closely resemble the processes of coagulation and fibrinolysis.

The plasminogen activation system

The plasminogen activation system, which regulates 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 synthesized in the liver and deposited in response to hyperpermeability. The formation of plasmin is essential for invasion and migration of endothelial cells into the tissue to be vascularized. 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 tumor cells[19;20]. A variety of cell types can bind components of the fibrinolytic system, including plasminogen[21], plasmin[22], uPA[23] 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 (MMP's) which further degrade the extracellular matrix. Activation of 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].

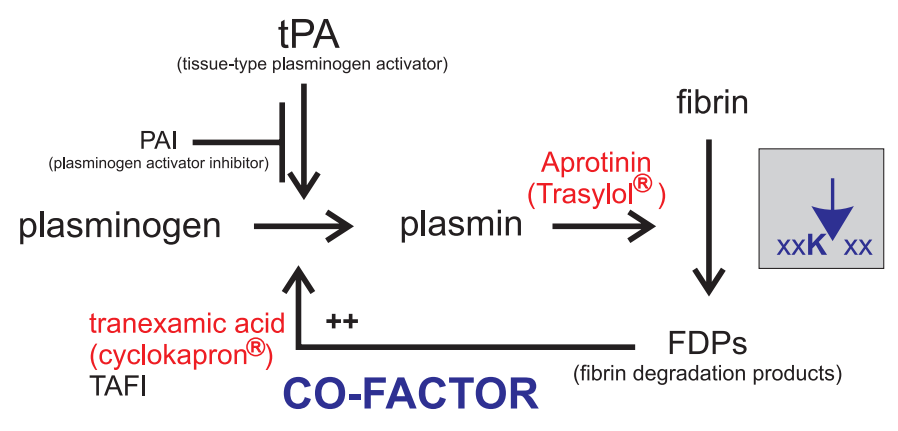
Lysine residues and lysine binding sites

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 (Figure 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].

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[38;46], a receptor expressed on endothelial cells. Since thrombomodulin expression can be upregulated by VEGF[47] 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[48-51]. Others have shown that TAFI is a repressor of plasminogen function during pulmonary clot lysis and peritoneal inflammation-induced leukocyte migration in mice with a compromised plasminogen background[52]. Taken together, TAFI is expected to control plasmin-mediated proteolysis of the provisional matrix during angiogenesis and tumor growth.

Figure 2.



The tPA-mediated plasminogen activation system. The inactive zymogen plasminogen can be activated into the serine protease plasmin by tissue-type plasminogen (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 carboxy-terminal lysines. Aprotinin (Trasylol®) and tranexamic acid (Cyclokapron®) inhibit fibrinolysis by blockage of plasmin activity and co-factor function of FDP respectively.

Anti-fibrinolytic therapy and cancer

Given plasmin's pivotal role in angiogenesis and tumor 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 I).

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 growth of human lung, ovarian and renal carcinoma's transplanted in nude mice. Inhibition was apparently caused by increased fibrin depositions at the advancing border of tumors due to reduced fibrinolytic activity[53]. Profound effects of tranexamic acid were seen on growth of lung, breast, hepatoma and ovarian carcinomas in other mice models[54;55]. In a study by Tanaka et al.[56] 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. In human mammary carcinoma and melanoma cells, tranexamic acid inhibited the binding of plasmin and plasminogen to the cell surface[57]. Another lysine analogue, ε-aminocaproic acid (εACA), decreased the tPA-mediated fibrinolytic activity of Co115 colon carcinoma cells *in vitro*[58]. Tumor-induced corneal angiogenesis could be significantly reduced by tranexamic acid and εACA[59]. Furthermore, εACA has been shown to inhibit glioma tumor growth in a mouse model[60] and *in vitro* angiogenesis[61]. Stabilization of active TAFI by εACA, determined by using a small substrate for TAFI, may give an additional inhibitory effect on fibrinolysis and angiogenesis[43]. 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 gr/day tranexamic acid[62]. In another study six out of eleven stage II or IV ovarian cancer patients responded to tranexamic acid therapy after surgical tumor debulking[63]. Taken together, since tranexamic acid has profound effects on angiogenesis and tumor growth and has no serious side effects 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[64].

Inhibitors of uPA

Inhibitors of uPA that either affect uPA activity or prevent uPA binding to its receptor have been successfully applicated *in vitro* and *in vivo*. Antibodies against uPA block tumor metastasis in the chorioallantoic membrane assay[65]. Furthermore, antibodies against uPA were used to inhibit metastasis and tumor growth in mice models[66-68]. Growth and formation of metastases of human cancer cell lines was inhibited after treatment with an uPA antagonist[69]. 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 tumor growth. Furthermore, establishment of primary tumors 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 SCID mice[69]. Min et al.[70] obtained comparable results using a similar approach. Ligation of the EGF domain of uPA to IgG resulted in a potent antagonist of uPA which inhibited capillary tube formation, bFGF induced neovascularization and B16 melanoma growth in syngenic mice. A similar construct was made by Lu et al.[71;72] who fused the amino-terminal fragment of uPA to human serum albumin. This construct inhibited *in vitro* tumor cell invasion and endothelial cell mobility and deformability. Inactive uPA, generated by PCR mutagenesis, that retains receptor binding reduced prostate cancer neovascularization and growth[73]. Prevention of *in vitro* tube formation in three-dimensional fibrin matrices was achieved with soluble uPAR and antibodies that inhibit uPA activity[61].

Table 1. Antiangiogenic and antitumorigenic compounds that deregulate the plasminogen activation system. 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 have been shown to have profound effects on either endothelial cell adhesion, migration, angiogenesis, metastasis or tumor growth.

Compound/Mechanism	Effect in vivo / in vitro
N-terminal fragment of uPA fused to IgG, uPA antagonist	arrests metastasis, inhibits establishment of primary tumors and micrometastases[69]
N-terminal fragment of uPA fused to IgG (m1-48Ig), uPA antagonist	*in vivo* suppression of basic fibroblast growth factor-induced neovascularization and B16 melanoma growth in syngenic mice[70]
N-terminal fragment of uPA ligated to HSA (ATF-HSA), uPA antagonist	*in vitro* inhibition of tumor cell invasion in matrigel, changes in cell morphology and remodeling of cytoskeleton[71;72]
Soluble uPAR	*in vitro* inhibition of human microvascular endothelial cells capillary formation in matrigel[61]
PAI-1 extended half-life, uPA inhibitor	inhibits prostate cancer xenografts[74]
p-Aminobenzamidine	inhibits growth of a human prostate tumor in mice and migration of endothelial cells in matrigel[74;75]
Amiloride	inhibits growth of a human prostate tumor in SCID mice[74]
Anti-catalytic uPA	inhibits the formation of lung metastasis of Lewis lung carcinoma and suppresses invasion of tumor cells through matrigel[66]
Anti-catalytic (human) uPA	no invasion of human carcinoma HEp3, however, no reduced incidence of distant metastasis in mice[68]
Anti-catalytic (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[65]
Inactive recombinant murine u-PA that retains receptor binding	inhibits prostate cancer neovascularization, metastasis and growth in rat[73]
Aprotinin (Trasylol®), inhibitor of plasmin and other serine proteases	inhibits invasion of endothelial cells on the human amniotic membrane[76], tube formation in matrix gels[61;77]and metastasis of Lewis lung carcinoma in mice[78]
Tranexamic acid (Cyclocapron®) and ε-aminocaproic acid (εACA), lysine-analogue which inhibits binding of plasminogen to its substrates	inhibits invasion and migration of endothelial cells on the human amniotic membrane[76], antiangiogenic in the cornea assay in rabbits[59] and *in vitro* angiogenesis[61]. Inhibits growth of V2 carcinoma in rabbits[79] of Lewis lung carcinoma in mice[56], of (human) tumors in mice[53-55;60;80]. Beneficial effects in humans have been reported[62;63;78;80-86]
α2-Antiplasmin, inhibitor of plasmin	inhibits *in vitro* tumor cell invasion through the human amniotic membrane[76]
Angiostatin and other fragments of plasmin, unknown mode of action (see text)	inhibits proliferation and migration of endothelial cells, neovascularization in the corneal assay and subcutaneous tumors[4;74;87-92]
Endostatin, unknown mode of action (see text)	inhibits primary tumor growth and metastasis[93-95], angiogenesis in CAM[96] and endothelial cell proliferation and migration[97], induces endothelial cell apoptosis[98]
Streptokinase and tPA, plasminogen activators which increase fibrinolysis	inhibits pulmonary tumor seeding in an animal model[99;100]
Potato carboxypeptidase inhibitor (PCI), inhibitor of TAFI, enhances fibrinolysis[51]	inhibits the growth of several human pancreatic adenocarcinoma cell lines in nude mice[101]

Others have used a physiological inhibitor of plasminogen activation, PAI-1, which half-life was extended by mutation[74]. Synthetic inhibitors of uPA activity like p-aminobenzamidine and amiloride showed a clear decrease in tumor-growth rate compared to untreated mice[74;75]. These results demonstrate that blockage of uPA by uPA inhibitors can reduce tumor size in experimental animals. Agents, such as aprotinin, which inhibit plasmin activity directly, can also inhibit metastasis[78].

Angiostatin

The observation that in some cases removal of a primary tumor in patients may lead to the rapid growth of previously undetected metastases[102;103] suggests that primary tumors make factors that may inhibit the outgrowth of distant tumors[104]. Based on this concept the angiogenesis inhibitor angiostatin was found[4]. Angiostatin is a proteolytic fragment of plasmin(ogen) and consists of kringle 1-4 of plasminogen. The molecule was purified from urine of tumor-bearing mice using lysine affinity chromatography. Systemic administration of angiostatin blocked neovascularization and growth of metastasis in the absence of the primary tumor. At higher doses, angiostatin can inhibit growth of primary tumors as well[89;90]. Continuous delivery of angiostatin has been shown to be more effective in inhibiting angiogenesis than bolus injection[64]. *In vitro*, angiostatin inhibits bFGF-induced endothelial cell proliferation and migration[4;105]. *In vivo* generation from plasmin(ogen) has been demonstrated and can be achieved by several proteases[106-110]. Angiostatin binds to the α/β-subunits of ATP synthase on the surface of endothelial cells causing cytolysis[111]. 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[112] thereby inducing changes in intracellular signalling[113]. Alternatively angiostatin may effect plasmin-mediated proteolysis through non-competitive inhibition of tPA activity[114]. However, also profibrinolytic effects have been described for kringle 1-3, which can block the interaction of plasmin with α2-antiplasmin[115]. Finally, angiostatin binds tissue factor and may regulate the formation of a provisional matrix through an effect on coagulation[116]. 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.

Endostatin

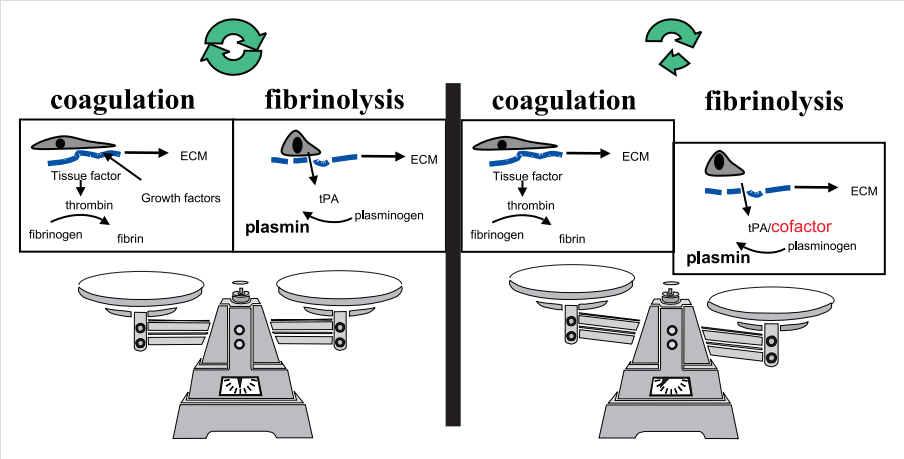
In 1997 a carboxy-terminal fragment of collagen XVIII, named endostatin, was identified that proved to be a potent inhibitor of endothelial cell proliferation and angiogenesis[97]. Endostatin was purified from conditioned media of hemangioendothelioma (EOMA) cells using a heparin affinity column. Like plasminogen and fibrinogen, collagen XVIII is made by hepatocytes[117]. Generation of endostatin can be achieved by cleavage of collagen by cathepsin L[118], matrilysin[119] or elastase[120]. Endostatin has distinct antiangiogenic and antitumoral activities in several animal models[121]. When administered to mice bearing Lewis lung carcinoma, T241 fibrosarcoma or B16F10 melanoma, recombinant mouse endostatin caused tumor regression without developing drug resistance[93]. However, contrary to this, no

antiangiogenic effect of endostatin was seen in other studies[122-125]. This still remaining paradox has lead to discussions about the efficacy of endostatin[126]. A complicating factor is that endostatin is used in a soluble, as well in an insoluble form. Although both forms can inhibit tumor growth, only the insoluble form induced complete regression of subcutaneous tumors[93]. Different structural forms of endostatin have distinct bioactivities and may therefore produce their antiangiogenic effects through distinct mechanisms[127;128] 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 chicken chorioallantoic membrane assay[96]. However, binding of endostatin to blood vessels was independent of heparan sulfate and endostatin did not compete with bFGF[129]. We have recently found a powerful molecular activity for endostatin that provides an explanation how endostatin may act as an antitumorigenic compound[127;128]. Because endostatin is a fragment of an extracellular matrix component, exerts its effects via the tumor vasculature and has a carboxy-terminal lysine, we examined whether endostatin can regulate plasmin formation. Using a subcutaneous colon carcinoma model we found that the antitumor activity of endostatin was completely abolished when mice were also treated with carboxypeptidase B. This suggests an important role for carboxy-terminal lysines in endostatin. The finding that endostatin purified from plasma of cancer patients lacks the carboxy-terminal lysine and is inactive in inhibiting endothelial cell proliferation[130] is in agreement with our findings. We established that endostatin binds plasminogen and stimulated tPA-mediated plasmin formation in a lysine-dependent manner. Like has 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 tumor growth (see below).

Can hyperfibrinolysis inhibit angiogenesis ?

Currently a well excepted 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 tumor will grow or stay dormant[4;104;131]. uPA and tPA are currently referred to as pro-angiogenic, whereas PAI is called an inhibitor of angiogenesis[5;132-135]. It has been suggested that angiogenic factors which induce angiogenesis induce endothelial expression of both uPA and PAI-1, with a slight excess in favor of the protease[136]. However, paradoxically PAI-1 has been correlated with a poor prognosis for many cancers (reviewed in [137]). 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[138]. 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[139]. We state that these “negative” regulators of angiogenesis are indispensable and stabilize this balanced process by limiting

Figure 3.



Angiogenesis is a continuous balanced process. Many tumors contain fibrin depositions[140;141] and elevated levels of plasminogen activators and PAI-1 (reviewed in [137]). In cancer patients levels of fibrinogen degradation products as well as complexes between plasmin and α_2-antiplasmin are elevated. This indicates an increased turn-over of fibrinogen and plasminogen. During angiogenesis the provisional matrix is continuously degraded and resynthesized, called remodeling. 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).

excessive proteolysis (see below). We propose an hemostasis model for angiogenesis. Our model considerates angiogenesis depending on a perfect balance of coagulation and fibrinolysis (Figure 3). Disturbation of this balance by inhibition, but also by overstimulation of fibrinolysis might prevent angiogenesis. This implicates 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 tumor 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 tumor growth (data not shown). Moreover, others have demonstrated *in vitro* that induction of plasminogen activation leads to endothelial cell detachment[142], inhibition of cell adhesion[143] or endothelial cell destruction[144]. Enhanced formation of plasmin, through administration of tPA or streptokinase (another plasminogen activator), also reduced pulmonary tumor seeding in an experimental animal model[99;100]. In addition, maspin, another stimulator of tPA, inhibits angiogenesis[145;146]. Our model may explain the observation that the absence of

PAI, which may lead to increased plasminogen activator activity and plasmin formation, prevents vascularization and tumor invasion[138]. Additionally, patients with peripheral tumors that are reduced by tumor necrosis factor alpha (TNFα) show elevated concentrations of tPA[147-149] and increased levels of plasmin-α2 antiplasmin (PAP)[148] and fibrin degradation products (FDP) [148;150]. Increased levels of PAP and FDP strongly suggest active fibrinolysis that correlates with tumor reduction. There might also be an additive effect of plasminogen activator activity on the migration of macrophages[151]. Inhibition of macrophage mobility will deprive tumor cells from growth factors. Similarly, excessive fibrinolysis might affect platelet interactions with fibrin in the tumor vasculature, thereby inhibiting angiogenesis[152]. Taken together, we propose that molecules that lead to excessive proteolysis in the tumor may be powerful antiangiogenic and antitumorigenic agents.

Cryptic fragments

An increasing number of proteolytic fragments, some of which may be generated naturally, are described with potent antiangiogenic activity. These include angiostatin[4], endostatin[97], antiangiogenic anti-thrombin III[3], restin[153], canstatin[154], kringle 5 of plasminogen[87] and thrombin fragment 1 and 2[156]. We found that fragments of fibrin (FDP) also possess antitumorigenic activity, possibly via a tPA-mediated mechanism similar to endostatin (chapter 8). At present it is unclear why these fragments are generated by tumors, 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 remodeling by controlling coagulation or fibrinolysis. These factors may only act antiangiogenic because they are administered at doses that excess endogenous levels, thereby disturbing strictly balanced proteolysis.

Concluding remarks

The interaction of endothelial cells and 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 tested in phase III clinical trials. A significant limitation of this approach is that the use of proteolytic inhibitors will never lead to removal of tumor stroma and therefore of the tumor itself. In fact, ultimately, the tumor 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 tumor regression. In this review we discussed that excessive localized 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 localized excessive proteolysis (“no grip, no growth”) will facilitate the development of an array of compounds that may be used in angiogenesis-related diseases.

Outline of the thesis

The research described in this thesis was aimed to further clarify the role of the plasminogen activation system in angiogenesis and cancer as a potential target for therapy. First, we investigated the role of components of this system in corneal angiogenesis and tumor growth. Second, we attempted to elucidate the working mechanism of the tumor growth inhibitor endostatin. Taken together, we addressed the following subjects:

1. The corneal angiogenesis model is widely used for (anti)angiogenic research. To what extent are components of the fibrinolytic system involved in corneal angiogenesis? In **chapter 2** we present the results of studies in mice deficient of plasminogen, plasminogen activator (tPA, uPA) or a regulator of plasminogen activation (PAI-1, TAFI).
2. The plasminogen system is activated during tumor growth. What is the role of a novel regulator of plasminogen activation, thrombin-activatable fibrinolysis inhibitor (TAFI), in tumor growth and metastasis? The effects of TAFI-deficiency on experimental tumor growth and metastasis formation are described in **chapter 3**.
3. Circulating markers of active coagulation and fibrinolysis and abnormal plasma levels of hemostatic proteins are often present in cancer patients, possibly leading to thrombotic and hemorrhagic events. This have prompted us to measure TAFI antigen and activity levels in plasma of patients with prostate cancer. In **chapter 4** the results are presented.
4. The working mechanism of the angiogenesis inhibitor endostatin is presently unknown. Endostatin is used in a soluble and insoluble form. Although both forms have been shown to inhibit tumor growth, the induction of complete tumor regression has only been reported for the insoluble form. Likely, these forms have different bioactivities. We determined the biological **(chapter 5)** and structural **(chapter 6,7)** differences between soluble and insoluble endostatin. Furthermore, we tested the hypothesis that endostatin acts through the induction of excessive proteolysis **(chapter 5)**.
5. We found that endostatin is a cofactor of tPA-mediated plasminogen activation. For a long time, fibrin fragments (fibrin degradation product, FDP) have been known as stimulators of tPA-mediated fibrinolysis. Can FDP also inhibit tumor growth? To address this question, we determined the effects of FDP treatment on endothelial cells and subcutaneous tumor growth **(chapter 8)**.
6. Could our observations be of value for the use of endostatin and other antiangiogenic fragments? In **chapter 9** we discuss the possible implications of our results.

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Chapter 2

The role of the fibrinolytic system in corneal angiogenesis

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Summary

The plasminogen activation system has been implicated in angiogenesis and angiogenesis-dependent diseases such as cancer, atherosclerosis and ocular diseases. The identification and development of inhibitors of angiogenesis offer new possibilities for the treatment of these diseases. To clarify the role of proteins involved in the regulation of fibrinolysis during corneal angiogenesis, we have studied corneal vessel formation in mice deficient for urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), plasminogen, plasminogen activator inhibitor-1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI). Our results corroborate earlier findings that angiogenesis in the mouse cornea is dependent on PAI-1 and plasminogen. The absence of tPA, uPA or TAFI did not affect the formation of new vessels in the cornea.

Introduction

Angiogenesis, the formation of new blood vessels from existing vasculature, is involved in normal development but also in a variety of pathologies such as cancer, atherosclerosis and ocular diseases[1;2]. During angiogenesis endothelial cells are activated and proteolytically dissect their way through the basement membrane, proliferate and migrate towards an angiogenic signal. Subsequently, the endothelial cells remodel into a new vessel. Proteolytic enzymes involved in matrix degradation include matrix metalloproteinases (MMP's) and the serine proteases of the plasminogen activation system, including tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) and plasmin[3;4]. The expression of these enzymes is induced by angiogenic factors, including VEGF and bFGF[5-7]. Plasmin contributes to angiogenesis directly by cleaving fibrin and other matrix components and indirectly by activating matrix-degrading metalloproteinases and angiogenic growth factors, such as transforming growth factor β[8], hepatocyte growth factor[9], vascular endothelial growth factor[10] and basic fibroblast growth factor[11].

Plasmin cleaves its substrates behind a lysine or arginine, which then become carboxy-terminal. These carboxy-terminal basic amino acid residues in fibrin provide new binding sites for plasminogen, facilitate its efficient activation by plasminogen activators and initiate a positive feedback loop[12-15]. Lysine analogues such as ε-aminocaproic acid (εACA) and tranexamic acid (Cyclokapron) or carboxypeptidase B-treatment efficiently prevent plasmin formation. At present, several reports have shown that plasminogen also interacts with proteins unrelated to fibrin. Similar to plasmin-cleaved fibrin, plasminogen-binding proteins like annexin II[16] and enolase[17], which are expressed by a variety of cell types, are involved in carboxy-terminal lysine or arginine-dependent plasminogen activation. The importance of carboxy-terminal basic amino acid residues in angiogenesis has been concluded from studies with ε-aminocaproic acid and tranexamic acid[18-21].

Plasminogen activation is regulated by thrombin-activatable fibrinolysis inhibitor (TAFI, also known as procarboxypeptidase U, -R, or plasma procarboxypeptidase B, EC 3.4.17.20)[22] and plasminogen activator inhibitor-1 (PAI-1)[23]. PAI-1 directly blocks the activity of tPA and uPA. TAFI is a carboxypeptidase B-type proenzyme that upon activation by thrombin[22], plasmin[24] or trypsin[25] removes carboxy-terminal lysines of fibrin and cellular binding sites of plasminogen causing reduced plasminogen binding and activation[26;27]. Thrombin-mediated activation of TAFI is strongly increased in the presence of both soluble and endothelial cell-bound thrombomodulin[28].

During the past decades, angiogenesis research has focussed on the isolation and characterization of inhibitors that might be of therapeutic value for the treatment of cancer and other angiogenesis-related diseases. The plasminogen activation system is a potential target of antiangiogenic treatment. For instance, the angiogenesis inhibitor angiostatin is a fragment of plasminogen[29] and endostatin is a stimulator of tPA-mediated plasminogen activation[30;31]. In addition, a variety of strategies to inhibit plasminogen activation and angiogenesis have been employed (reviewed in [32]). The identification of various

angiogenesis inhibitors has prompted the development of angiogenesis models to test their anti- or proangiogenic efficacy. At present, the corneal angiogenesis assay is widely used and considered one of the best *in vivo* models to study angiogenesis because of the avascularity of the cornea[2]. Here we studied the role of plasminogen, uPA, tPA, PAI-1 and TAFI in corneal neovascularization using plasminogen$^{-/-}$, uPA$^{-/-}$, tPA$^{-/-}$, PAI-1$^{-/-}$ and TAFI$^{-/-}$ mice.

Materials and methods

Animals

Mice deficient for uPA, tPA, plasminogen, PAI-1 and littermate wildtype controls (i.e. identical phenotype apart from deleted gene) were kindly provided by Prof. P. Carmeliet (Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biology, Leuven, Belgium). These and TAFI-deficient mice were described previously[33-36]. All animals were maintained under specified pathogen free conditions, received food and water *ad libitum* and were kept on a 12-hour light/dark cycle. All experiments were performed in accordance with guidelines of the University's Animal Experimental Committee, University Medical Center Utrecht, The Netherlands.

Corneal angiogenesis assay

The corneal angiogenesis assay was performed as described previously[37]. Mice (6-8 weeks of age) were anaesthetized by intraperitoneal administration of a mixture of Hypnorm (0.3 mg/mouse; Janssen-Cilag, Brussels, Belgium) and Dormicum (12.5 mg/mouse, Roche, Brussels, Belgium). In addition, corneas were anaesthetized by local administration of 4 mg/ml oxybuprocaine (0.4%) eye drops. Corneal micro pockets were created with surgical blade #10, a modified keratotome and a pair of microscopic forceps. Micro pellets were constructed as described previously[37]. Briefly, a suspension of 100 ng bFGF (Life technologies, Rockville, MD), sucrose aluminium sucralfate and Hydron (IFN Sciences New Brunswick, NJ) was prepared and applied to a sterilized nylon mesh (approximate pore size 0.4 x 0.4 mm). This suspension was allowed to dry, after which the fibers of the mesh were separated under sterile conditions; same size pellets were selected for implantation. The pellets were deposited into the micro pocket at a distance of 0.9-1.1 mm from the limbus vasculature. Eyes were treated with aknemycin ointment to prevent infection and dehydration of the cornea. Six days after pellet implantation, mice were anaesthetized as described above and correct position (0.9-1.1 mm from the limbus) of the pellet was confirmed in all eyes. Angiogenesis in the cornea was measured as corneal neovascular surface area (A). The surface area of newly formed blood vessels was calculated using the formula 0.2 x π x maximal vessel length (VL) x the contiguous circumferential zone of neovascularization (CN = clock hours, 1 clock hour equals 30 degrees of arc)[38].

Statistical analysis

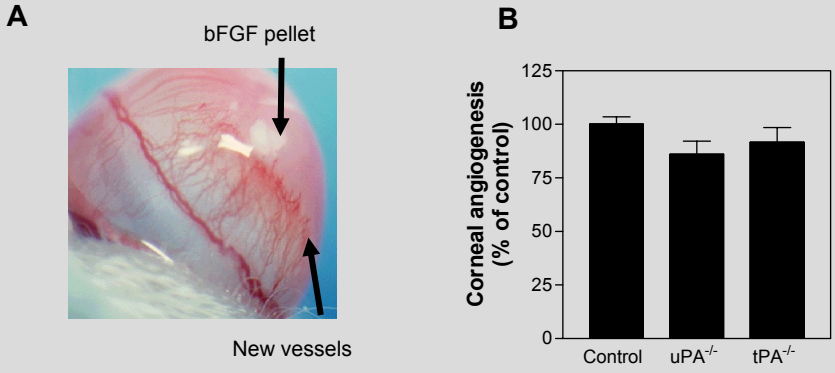
The area of vessel growth in control mice was considered 100%. Neovascularization in other groups was expressed as percentage of that in control mice. All data were expressed as the normalized mean ± SEM. The significance of differences was determined by unpaired student's t-test; $P < 0.05$ was considered to be statistically significant.

Results

Corneal neovascularization in mice deficient for plasminogen activators, uPA and tPA

Angiogenic growth factors such as bFGF and VEGF induce the expression of plasminogen activator (uPA and tPA) by endothelial cells[5-7]. To investigate the effects of plasminogen activator (uPA and tPA)-deficiency on bFGF-induced corneal angiogenesis we performed the cornea neovascularization assay in uPA-deficient, tPA-deficient and control mice. In response to bFGF, new blood vessels formed from the limbal plexus reaching the implanted pellet six days after implantation (Figure 1a). Triangular vessel growth was observed from the limbus (base) towards the pellet (apex). In all uPA- and tPA-deficient mice, bFGF induced the formation of new blood vessels reaching the pellet at day 6 (Figure 1b). In uPA-deficient mice, mean vessel area was slightly but not significantly decreased when compared to control mice ($uPA^{-/-}$: 86.0 ± 6.1%, n = 5; control: 100.0 ± 3.5%, n = 24, p = 0.36). The observed slight decrease in vessel area was due to a decreased number of clock hours. In tPA-deficient mice, the area of newly formed vessels was similar to that in control mice ($tPA^{-/-}$: 91.7 ± 6.8%, n = 6; control: 100.0 ± 3.5%, n = 24, p = 0.53).

Figure 1.

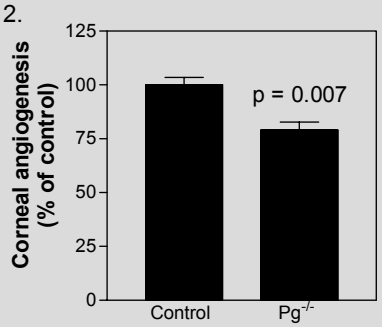


Corneal neovascularization in $uPA^{-/-}$, $tPA^{-/-}$ and control mice. (A) Photograph of a control mouse cornea 6 days after bFGF-coated pellet implantation. (B) bFGF-induced vessel formation in corneas of $uPA^{-/-}$ (n = 5), $tPA^{-/-}$ (n = 6) and control (n = 24) mice was analyzed 6 days after pellet implantation and quantified. Corneal angiogenesis was unaffected in the absence of tPA or uPA. Values are the normalized mean ± SEM.

Corneal neovascularization in mice deficient for plasminogen

Both uPA and tPA can activate plasminogen. Therefore, the observed progression of vessel growth in mice lacking uPA and tPA could be due to complementary bioactivity of these enzymes. In addition, plasminogen activation is not necessarily dependent on the presence of tPA or uPA since alternative pathways exist that can activate plasminogen into plasmin[39;40]. To directly determine the role of plasmin(ogen) in corneal angiogenesis, we analyzed corneal vessel growth in plasminogen-deficient mice (Figure 2). In plasminogen-deficient mice, we observed a significant reduction in vessel growth compared to control mice (plasminogen$^{-/-}$: 79.0 ± 3.8%, n = 6; control: 100.0 ± 3.5%, n = 24, p = 0.007). These results indicate a functional role for plasminogen in corneal angiogenesis.

Figure 2.

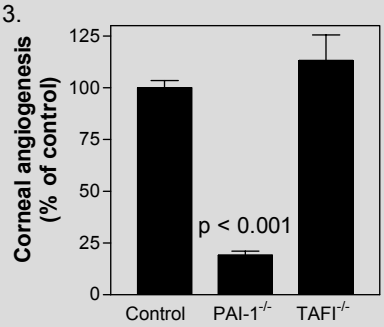


Corneal neovascularization in plasminogen$^{-/-}$ and control mice. bFGF-induced vessel formation in corneas of plasminogen$^{-/-}$ (n = 6) and control (n = 24) mice was analyzed 6 days after pellet implantation and quantified. Plasminogen-deficiency significantly reduced corneal angiogenesis. Values are the normalized mean ± SEM. P-values are indicated in the figure.

Corneal neovascularization in mice deficient for inhibitors of plasminogen activation, TAFI and PAI-1

Plasminogen activation is regulated by plasminogen activator inhibitor-1 and thrombin-activatable fibrinolysis inhibitor (TAFI). PAI-1 deficiency is associated with significantly decreased corneal vessel formation (PAI$^{-/-}$: 19.0 ± 2.0%, n = 6; control: 100.0 ± 3.5%, n = 24, p < 0.001) (Figure 3). Possibly, uncontrolled plasminogen activation caused by the absence of PAI-1 could lead to reduced angiogenesis. To further explore this hypothesis we examined corneal neovascularization in mice lacking another inhibitor of plasminogen activation, TAFI (Figure 3). Corneal vessel formation in response to bFGF in TAFI-deficient mice was similar to vessel growth observed in controls (TAFI$^{-/-}$: 98.1 ± 11.2%, n = 5; control: 100.0 ± 3.5%, n = 24, p = 0.17).

Figure 3.



Corneal neovascularization in PAI-1$^{-/-}$, TAFI$^{-/-}$ and control mice. bFGF-induced vessel formation in corneas of PAI$^{-/-}$ (n = 6), TAFI$^{-/-}$ (n = 5) and control (n = 24) mice was analyzed 6 days after pellet implantation and quantified. Corneal angiogenesis was highly attenuated in mice lacking PAI-1 and normal in TAFI$^{-/-}$ mice. Values are the normalized mean ± SEM. P-values are indicated in the figure.

Discussion

The corneal neovascularization model is frequently used to study angiogenesis *in vivo* and to test potential angiogenesis inhibitors. Numerous studies have indicated an important role of the fibrinolytic system in disease-related angiogenesis. In the present study we investigated the role of different components of the fibrinolytic system in corneal angiogenesis. Plasminogen is present in the human cornea[41], maintains the normal mouse cornea by preventing from ligneous conjunctivitis[42] and is involved in corneal wound healing[43]. In addition, mechanically wounding of the cornea induced the expression of tPA and uPA by the corneal epithelium in rabbits[44]. We found that plasminogen, tPA, uPA and TAFI are not essential for efficient corneal vessel formation. However, plasminogen-deficiency caused a significant reduction of corneal angiogenesis. Angiogenesis was largely reduced in the absence of PAI-1 but was normal in TAFI-deficient mice.

uPA proteolytic function is considered to be important for angiogenesis. Using an uPA-peptide that blocks the interaction of uPA with its cellular receptor, uPAR, McGuire et al.[45] have shown inhibitory effects in a mouse model of ischemia-induced retinal neovascularization. Others have shown that the uPAR-binding domain of uPA is a potent inhibitor of bFGF-induced neovascularization in a subcutaneously implanted matrigel plug and B16 melanoma growth in mice[46]. In addition, prostaglandin E1-induced corneal angiogenesis in rabbits was decreased upon intraperitoneal treatment with amiloride, an inhibitor of uPA activity[47]. Based on these antiangiogenic effects of compounds that block uPA function, one would expect that corneal angiogenesis is largely diminished in mice lacking uPA. Choroidal neovascularization is blocked in uPA-deficient mice[48]. Recently, Oh et al.[49] showed a slight reduction of corneal vessel formation in uPA-deficient mice. However, we show here and others have done previously[50], that corneal angiogenesis is normal in uPA-deficient mice. These results therefore indicate that the antiangiogenic effects of uPA inhibitors *in vivo* may be largely due to other activities than direct uPA inhibition.

tPA is stored in endothelial cells of the microvasculature[51] and secreted upon activation by angiogenic factors[7]. tPA has been implicated in capillary formation *in vitro* in collagen[52] but not in fibrin[53]. In addition, capillary sprouting in collagen and matrigel of vascular explant cultures obtained from tPA- and plasminogen-deficient mice was almost completely abrogated[54]. Furthermore, tPA is required for efficient vascularization of pancreatic tumors[55] and is essential in choroidal neovascularization[48]. In contrast, tPA-deficiency did not affect angiogenesis in collagen induced by malignant keratinocytes in mice[56]. It has been suggested that tPA has a role in protecting newly formed blood vessels that would be prone to extracellular matrix-induced coagulation[4]. However, we did not observe an effect on corneal angiogenesis in tPA-deficient mice.

Possibly, like has been shown previously to occur during vascularization induced by malignant keratinocytes[56], a compensatory mechanism that upregulates tPA in uPA-deficient mice and uPA in tPA-deficient mice may exist. Moreover, plasminogen activation is not necessarily dependent on the presence of tPA or uPA but might be compensated by other

enzymes such as coagulation factor XIIa[39] or kallikrein[40]. Therefore we directly investigated plasminogen function in corneal angiogenesis using plasminogen-deficient mice. Our results suggest a role, albeit minor, of plasmin-mediated matrix degradation during vessel formation in the cornea. Our data confirm results from a recent study by Oh et al.[49] showing that plasminogen-deficiency significantly reduced corneal neovascularization. Remarkably, although it is widely accepted that plasminogen is an important mediator of angiogenesis, this process can occur in the absence of plasminogen.

In previous studies, we have shown that corneal neovascularization was almost completely inhibited by continuous treatment with angiostatin, a fragment of plasminogen[37]. Stack et al. [57] have shown that angiostatin inhibited invasion of endothelial and melanoma cells *in vitro* by blocking tPA-mediated matrix-enhanced plasminogen activation. Our results suggest that the antiangiogenic effect of angiostatin *in vivo* may not only be mediated through inhibition of plasminogen function.

Plasminogen activation is negatively controlled by both PAI-1 and TAFI. It has previously been shown that PAI-1 has an important function in the growth of human melanoma[58] and mouse fibrosarcoma[59]. These reports further showed that the lack of PAI-1 directly affected angiogenesis in the cornea[59] and a matrigel implant[58]. In a model of vessel wall remodeling after oxidative arterial injury, neovascularization was defective in the absence of PAI-1[60]. Others have shown that PAI-1 is essential for subretinal choroidal angiogenesis induced by laser photocoagulation[61] and tumor cell induced angiogenesis in collagen in mice[56]. Based on experiments in mice deficient of both PAI-1 and vitronectin these authors concluded that PAI-1 is mainly involved in the prevention of excessive proteolysis. Interestingly, increased plasmin activity has been associated with detachment of endothelial[62] and smooth muscle cells[63], inhibition of keratinocyte adhesion[64], endothelial cell destruction[65] or regression of capillary tubes[66] *in vitro*.

We hypothesized that similar to the effects in mice lacking PAI-1, TAFI-deficiency would also lead to increased proteolysis and aberrant angiogenesis. However, in contrast to PAI-1, the absence of TAFI did not affect vessel formation in the cornea. We propose two possible explanations for the lack of effects in the absence of TAFI. First, TAFI is activated but proteins that neutralize increased plasminogen activation in TAFI-deficient mice, including PAI-1, α_2-antiplasmin or other carboxypeptidases, compensate for the absence of TAFI. Second, TAFI is not activated and does not regulate plasminogen activation in corneal angiogenesis. Alternatively, increased plasminogen activation in the absence of TAFI does not influence corneal angiogenesis. At present, no tools are available to measure activated TAFI in blood or tissues. Our observation that TAFI-deficiency does not affect corneal angiogenesis support a role for PAI-1 different from regulation of plasminogen activation. Besides its anti-proteolytic activity, PAI-1 is also involved in vitronectin-mediated cellular adhesion[67].

Taken together, we show that corneal angiogenesis is independent of uPA- or tPA-mediated plasminogen activation. Although plasmin was not essential for vessel formation, plasminogen-deficiency had significant effects. Corneal neovascularization was largely

dependent on PAI-1 but unaffected in the absence of another regulator of plasminogen activation, TAFI.

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Chapter 3

Tumor growth and metastasis are not affected in Thrombin-activatable Fibrinolysis Inhibitor deficient mice

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Summary

Many studies have indicated that the plasminogen activation system may have a prominent role in cancer. Activation of the zymogen plasminogen into the serine protease plasmin by plasminogen activator is mediated by carboxy-terminal basic amino acids in fibrin, including lysines and arginines. Activated thrombin-activatable fibrinolysis inhibitor (TAFI) is a circulating carboxypeptidase B-type enzyme that removes carboxy-terminal lysine or arginine residues in fibrin, resulting in decreased plasminogen activation and attenuated fibrinolysis. To directly determine whether TAFI is involved in primary tumor growth and metastasis formation we examined the effects of TAFI-deficiency on subcutaneous growth and experimentally or spontaneously induced pulmonary metastasis formation of different tumor cell types in mice. In all tumor models TAFI-deficiency did not affect the formation and growth of primary and metastasized tumors.

Introduction

Apart from its role in fibrinolysis the plasminogen activation system is implicated in tumor development and metastasis of experimental tumors and human cancers[1-3]. Increased levels of plasminogen activator, plasmin-α2-antiplasmin, plasminogen activator inhibitor (PAI-1) and fibrin degradation products have been observed in plasma of cancer patients and these components are of prognostic value in human cancer[1;4-8]. The plasminogen activation system controls cell migration and invasion by plasmin-mediated matrix proteolysis. A major plasmin substrate, fibrin, is found in the provisional matrix of tumors[9;10]. Moreover, fibrin is important for metastasis of circulating tumor cells[11-13]. Plasmin activity is not restricted to the dissolution of extracellular matrix. In addition to this, plasmin regulates tumor growth and metastasis by the activation of matrix metalloproteases (MMP) and growth factors such as transforming growth factor β[14] and hepatocyte growth factor[15], mobilises vascular endothelial growth factor (VEGF)[16] and basic fibroblast growth factor (bFGF)[17] and could play a role in the formation of plasminogen breakdown products with antiangiogenic properties, including angiostatin and kringle 5[18]. Taken together, substantial evidence points to an important role of the plasminogen activation system in tumor progression.

Plasmin, that is formed through activation of the zymogen plasminogen by plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), is a serine protease that cleaves its substrates behind a basic amino acid (lysine or arginine). The generally free carboxy-terminal lysines or arginines provide high affinity binding sites for plasminogen and facilitate efficient activation by plasminogen activators[19-24]. This subsequently leads to the formation of novel plasminogen binding sites resulting in an amplification loop for plasminogen activation[25]. In addition, the carboxy-terminal lysine on the A-chain of urokinase promotes the activation of plasminogen[26]. Hence, lysine analogues such as ε-aminocaproic acid (εACA) and tranexamic acid (cyclokapron) efficiently prevent plasmin formation[27-29]. Besides cleaved fibrin or other plasmin-cleaved extracellular matrix components, a variety of cell types also have carboxy-terminal lysine-dependent binding sites for plasminogen, like annexin II[30] and alpha-enolase[31], that regulate plasminogen activation. The possible importance of carboxy-terminal lysine residues in cancer can be inferred from studies with εACA or cyclokapron (reviewed in[32]). These lysine analogues inhibit angiogenesis, tumor cell metastasis and primary tumor growth. Moreover, beneficial effects of these compounds have been reported in cancer patients[33;34].

Activated thrombin-activatable fibrinolysis inhibitor (TAFI, also called carboxypeptidase U, R or plasma procarboxypeptidase B, EC 3.4.17.20) is a carboxypeptidase B-type enzyme that regulates plasminogen activation by cleaving carboxy-terminal arginyl and lysyl residues from partially degraded fibrin, thereby attenuating plasminogen activation[35;36]. *In vitro*, TAFI can be activated by trypsin[37], plasmin[38] and thrombin[35]. Activation of TAFI by plasmin can be improved by heparin[39]. Activation of TAFI by thrombin is increased 1250-fold in the

presence of thrombomodulin[40;41]. Thrombomodulin is expressed on endothelial cells and in tumors[42-46] and is a prognostic factor in human cancer[47;48].

Recently, we have shown that TAFI regulates cutaneous wound healing[49], a process that is dependent on activation of the plasminogen system[50]. In mice lacking TAFI, skin wound closure was inappropriately organized and slightly delayed and epithelial migration and invasion were altered. Given the importance of the plasminogen activation system in tumor growth and metastasis, we hypothesized that TAFI also plays a regulatory role in cancer growth and could be a potential new target for anti-cancer drugs. In the present work, we have investigated the role of TAFI in subcutaneous tumor growth and experimental- and spontaneous metastasis formation using TAFI-deficient mice.

Materials and methods

Mice

$TAFI^{-/-}$ (generated as described in[49]) and $TAFI^{+/+}$ littermate mice, 6-8 weeks of age, were maintained under standard conditions, kept on a 12-h light/dark cycle and received food and water *ad libitum*. Mice used for experiments with B16-BL6 melanoma cells were inbred into the C57BL/6 background (Harlan, Leicestershire, England) for four generations. Mice used in all other experiments were inbred into C57BL/6 for six generations. Genotypes of the mice were determined by polymerase chain reaction analysis of DNA obtained from ear biopsies and verified by ELISA using anti-mouse TAFI antibodies. Experiments were performed according to the guidelines of the Utrecht Animal Experimental Committee, University Medical Center Utrecht.

Tumor cell lines

The B16-BL6 melanoma cell line was kindly provided by Dr. K.L. Sim (Entremed Inc., Rockville, Maryland). The T241 fibrosarcoma and Lewis lung carcinoma (LLC) cell lines were a generous gift of Dr. T. Udagawa (Children's Hospital, Harvard Medical School, Boston, Massachusetts). B16-BL6 and LLC cells were cultured *in vitro* by subconfluent passage in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Invitrogen Corporation, UK) containing 10% fetal calf serum (FCS), L-glutamine and antibiotics in a humidified 10% CO_2 environment. The T241 fibrosarcoma cells were maintained in McCoy's 5A modified medium (Gibco BRL, Invitrogen Corporation, UK) supplemented with 10% FCS, antibiotics, L-glutamine, MEM non-essential amino acids, MEM amino acids and MEM vitamins in a humidified 10% CO_2 environment. These tumor cell lines were derived from C57BL/6 mice and thus should be H2 histocompatible with all mice used in this study (TAFI-deficient mice were derived from C57BL/6 and 129 mice both of which are homozygous for the b allele at the H2 locus).

Growth of subcutaneous tumors

Confluent B16-BL6 melanoma cells were washed with phosphate-buffered saline (PBS) and incubated with 0.25% trypsin, 0.53 mM EDTA solution (Gibco BRL, Invitrogen Corporation,

UK) for 1 minute at 37°C. Trypsin was inactivated by the addition of 5 ml DMEM containing 10% FCS. The volume was brought to 50 ml with PBS. After centrifugation the cells were washed two times with 50 ml PBS. 100 µl of resuspended cells (1.0 x 10^7/ml PBS) were injected subcutaneously between the shoulder blades ~3 cm from the tail. T241 fibrosarcoma and Lewis lung carcinoma were propagated in C57BL/6 mice. Mice with 600-1200 mm^3 tumors were sacrificed and cleaned with betadine and ethanol. Tumor tissue was excised and a suspension of tumor cells in 0.9% saline was made by passage of viable tumor tissue through a sieve and a series of sequentially smaller needles of a diameter of 22-30 gauge. The final concentration of trypan blue negative tumor cells was adjusted to 1 x 10^7 cells/ml and 100 µl was injected subcutaneously. Palpable tumors were measured using a calliper and tumor volume was calculated using the formula: $width^2$ x length x 0.52.

Experimental pulmonary metastasis formation

Confluent B16-BL6 melanoma cells were washed with phosphate-buffered saline (PBS) and incubated with 0.25% trypsin, 0.53 mM EDTA solution (Gibco BRL, Invitrogen Corporation, UK) for 1 minute at 37°C. Trypsin was inactivated by the addition of 5 ml DMEM containing 10% FCS. The volume was brought to 50 ml with PBS. After centrifugation the cells were washed two times with 50 ml PBS. The cells were resuspended in 5 ml PBS and cell viability was determined using trypan blue. Using a 27-gauge needle, 5 x 10^4 melanoma cells in 200 µl PBS were injected into the lateral tail vein. Fourteen days after inoculation of tumor cells all mice were euthanized by CO_2 inhalation. Lungs were isolated and placed in 4% neutral buffered formalin. The lungs were separated into individual lobes and the number of surface metastatic foci was counted under a dissecting microscope.

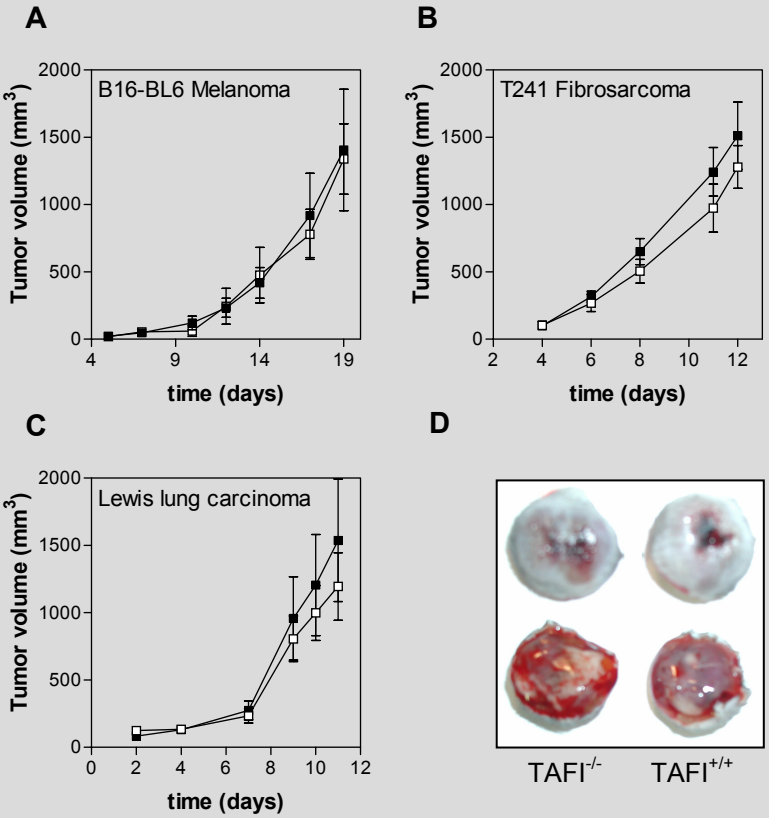
Spontaneous pulmonary metastasis formation

Eleven days after subcutaneous LLC transplantation, the mice were anaesthetized using a mixture of Hypnorm® (0.3 mg/mouse intraperitoneal; Janssen-Cilag, Brussels, Belgium) and Dormicum® (12.5 mg/mouse intraperitoneal; Roche , Brussels, Belgium). The skin overlying the tumor was cleaned with betadine and ethanol. Primary tumors were surgically removed and the incision was closed using vicryl 5/0. Fourteen days later, the lungs and lymph nodes were isolated. Surface metastases were highlighted by incubation in Bouin's fixative for 24 h. The lungs were separated into individual lobes and the number of surface metastatic foci was counted under a dissecting microscope.

Histologic analysis

Subcutaneously located tumors and lungs were embedded in paraffin after fixation in 4% neutral buffered formalin. 3µ sections were stained with hematoxylin and eosin according to established methods and evaluated for the presence of histological differences between either subcutaneous and pulmonary tumor deposits and/or between TAFI-deficient and wildtype mice.

Figure 1.



Effect of TAFI-deficiency on primary tumor growth. 1 x 10^6 B16-BL6 melanoma (A), T241 fibrosarcoma (B) or Lewis lung carcinoma (C) cells were injected into the subcutaneous dorsa of mice and tumor volume was determined by calipation. No significant differences in tumor growth were observed between TAFI-deficient and wildtype mice. □, $TAFI^{-/-}$; ■, wildtype. (D) Representative example of subcutaneous LLC of a TAFI-deficient mouse (left panel) and a wildtype mouse (right panel) 11 days after transplantation. Upper panel: skin side facing up, Lower panel: basal side facing up.

Results

Effect of TAFI-deficiency on subcutaneous tumor growth

To determine the role of TAFI in primary tumor growth B16-BL6 melanoma, T241 fibrosarcoma or Lewis lung carcinoma (LLC) cells were injected subcutaneously into the dorsal skin of TAFI-deficient and wildtype mice. All mice had palpable tumors by day 5. Tumor burdens derived from all three cell lines did not differ significantly in TAFI-deficient from those in wildtype mice (Figure 1a-c). All three tumor types grew in parallel regardless of TAFI-genotype. At day 19, B16-BL6 tumors in TAFI-deficient mice were 1406 ± 451 mm^3 (mean ± SEM, n=5) compared to 1339 ± 262 mm^3 in wildtype mice (n=4; p = 0.73, Mann-Whitney U test) (Figure 1a). The tumor volumes of T241 fibrosarcoma and LLC were 1280 ± 159 mm^3 (n=5, $TAFI^{-/-}$) compared to 1513 ± 249 mm^3 at day 12 (n=9, $TAFI^{+/+}$; p = 0.36, Mann-Whitney U test) (Figure 1B) and 1181 ± 225 mm^3 (n=10, $TAFI^{-/-}$) compared to 1375 ± 417 mm^3 at day 11 respectively (n=7, $TAFI^{+/+}$; p = 0.74, Mann-Whitney U test) (Figure 1c). In contrast to tumors derived from B16-BL6 melanoma and T241 fibrosarcoma cells, the growth of LLC primary tumors was associated with overlying skin redness caused by progressive ulceration. This was independent of TAFI-genotype (Figure 1d). Thus, TAFI-deficiency did not significantly affect the onset and kinetics of primary tumor growth.

Establishment of experimental lung metastases in TAFI-deficient mice

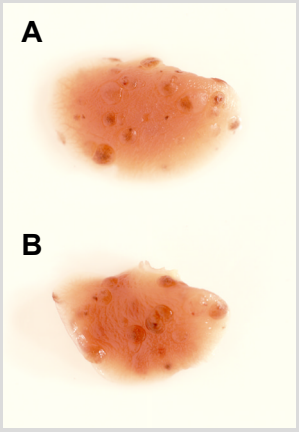
Experimental metastasis formation of tumor cells is dependent on the formation and subsequent removal of thrombi consisting of tumor cells, platelets and fibrin[12]. To determine whether these processes are influenced by TAFI we injected 5 x 10^4 B16-BL6 melanoma cells into the tail vein of TAFI-deficient and wildtype mice and counted the number of surface pulmonary nodules at day 14 following injection. B16-BL6 melanoma cells were able to metastasize and establish visible pulmonary tumor foci in both TAFI-deficient and wildtype mice (Figure 2a,b). No significant difference between the number ($TAFI^{-/-}$: 92 ± 25, n = 11; $TAFI^{+/+}$: 89 ± 20, n = 6; p = 0.88, mean ± SEM, Mann-Whitney *U* test) (Figure 2c) and size distribution (n = 449 foci in $TAFI^{-/-}$, n = 413 foci in $TAFI^{+/+}$; p = 0.57, Kolmogorov-Smirnov test) of metastases in TAFI-deficient and wildtype mice was observed (Figure 2d).

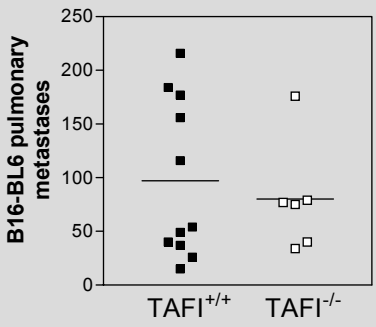
Establishment of spontaneous metastases in TAFI-deficient mice

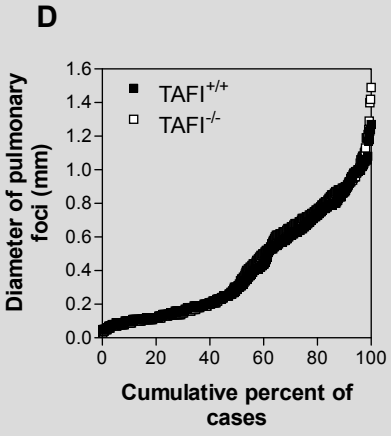
To determine the role of TAFI in spontaneous metastasis formation we transplanted Lewis lung carcinoma tumors subcutaneously and analyzed metastasis formation in lungs and regional lymph nodes of TAFI-deficient and wildtype mice 14 days after primary tumor resection. We did not observe regrowth of the primary tumor. The formation of lymph node metastases was clearly visible 14 days after primary tumor resection. Visual inspection revealed that lymphatic spread of tumor cells was macroscopically present in 7 of 9 (78%) TAFI-deficient mice compared to 5 of 5 (100%) wildtype mice (p = 0.5, χ^2 analysis). Lung metastases were present in TAFI-deficient and wildtype mice (Figure 3a), suggesting that TAFI is not essential for lymphatic or hematogenous metastasis. Quantification of the

number (TAFI$^{-/-}$: 17 ± 6, n = 9; TAFI$^{+/+}$: 20 ± 8, n = 5; p = 0.80, mean ± SEM, Mann-Whitney U test) (Figure 3c) and size distribution (n = 52 foci in TAFI$^{-/-}$, 122 foci in TAFI$^{+/+}$; p = 0.24, Kolmogorov-Smirnov test) of metastatic foci in lungs of TAFI-deficient and wildtype mice revealed no significant difference (Figure 3d). These results indicate that TAFI does not contribute to the efficiency of this tumor to colonize lung tissue.

Figure 2.

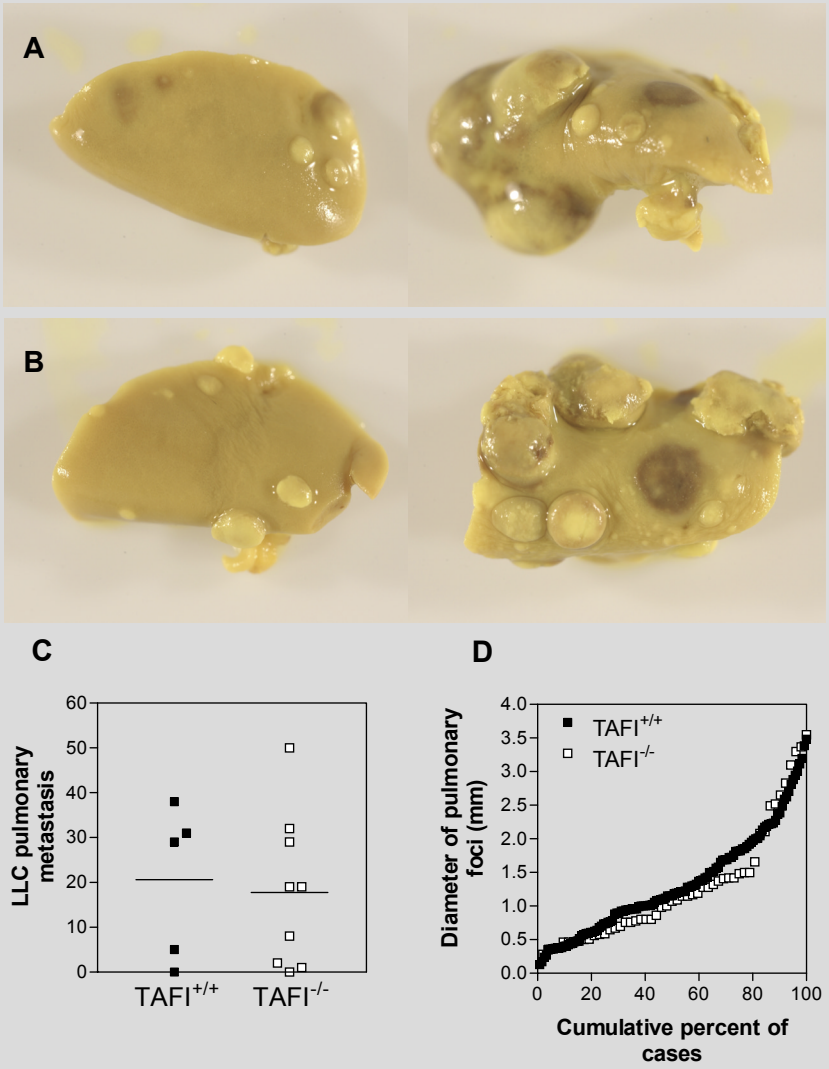






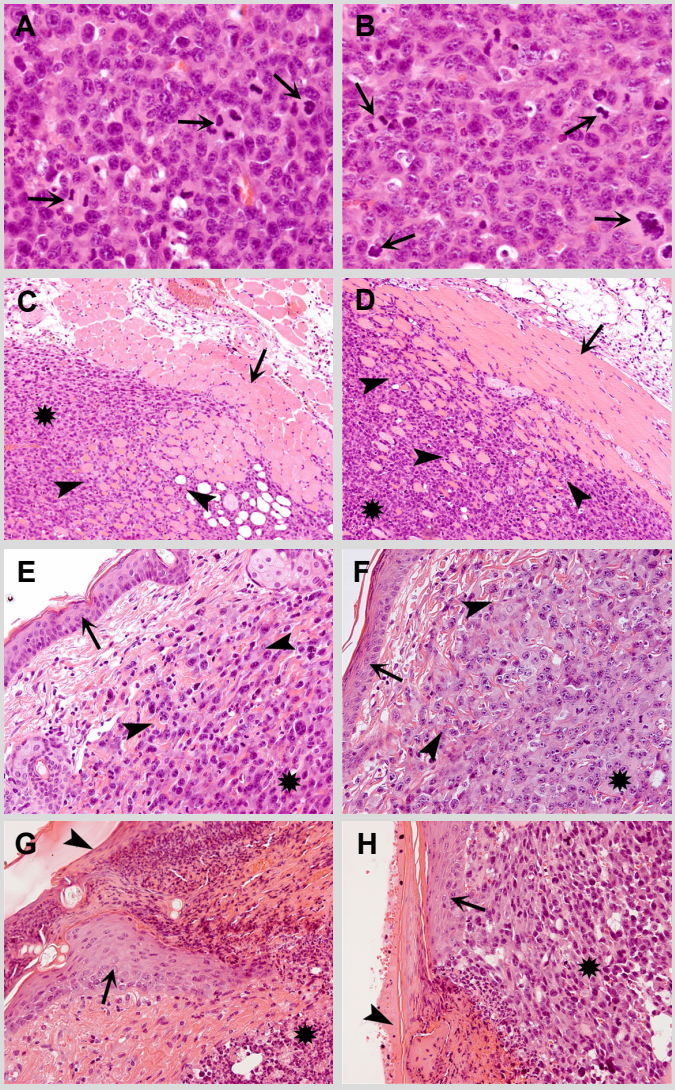
Effect of TAFI-deficiency on experimental pulmonary metastasis formation. 5 x 10^4 B16-BL6 melanoma cells were injected intravenously in TAFI-deficient and wildtype mice by tail vein injection. Lungs were isolated after 14 days, fixed and pulmonary metastasis formation was quantified by counting the number of tumor foci. (A,B) Representative examples of lungs with metastatic foci of B16-BL6 melanoma in wildtype (A) and TAFI-deficient mice (B) 14 days after intravenous injection. (C) The number of pulmonary nodules in TAFI-deficient and wildtype mice was not significantly different. (D) TAFI-deficiency does not affect the size distribution of pulmonary metastases of B16-BL6.

Figure 3.



Effect of TAFI-deficiency on spontaneous pulmonary metastasis formation. TAFI-deficient and wildtype mice underwent primary tumor resection 11 days after subcutaneous transplantation of LLC. After 14 days the lungs were removed and fixed in Bouin's fixative. (A,B) Representative examples of metastatic pulmonary foci 14 days after tumor resection, indicating the presence of both small and large tumor nodules in wildtype (A) and TAFI-deficient (B) mice. (C) Quantification of the number of pulmonary metastatic foci revealed no significant difference between TAFI-deficient and wildtype mice. (D) Size distribution of individual metastatic foci is not altered by TAFI.

Figure 4.

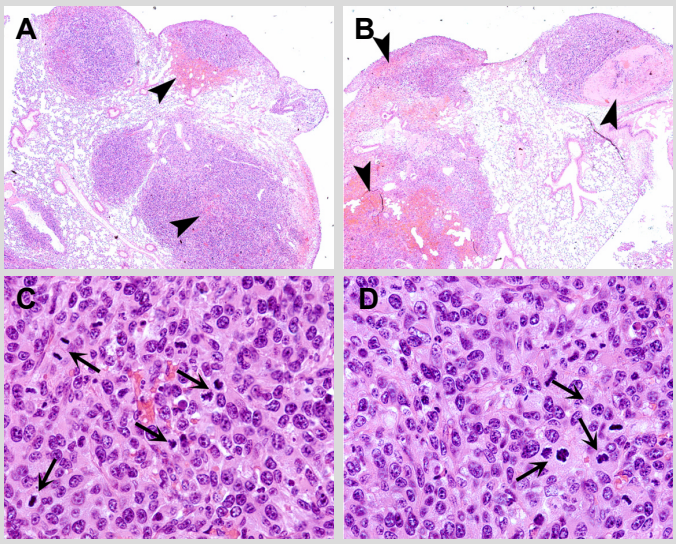


Histological analysis of primary tumors in TAFI-deficient (A,C,E,G) and wildtype (B,D,F,H) mice. Primary tumors of LLC were isolated 11 days after transplantation, paraffin embedded, sectioned and stained with hematoxylin/eosin. (A,B) Tumors in TAFI-deficient and wildtype mice both consist of closely packed anaplastic cells of different sizes with ample cytoplasm and show numerous normal as well as abnormal mitoses (arrows). (C,D) Tumors in both genotypes exhibited diffuse invasion into and destruction of adjacent muscle tissue. Arrows indicate normal muscle, arrowheads indicate tumor cell invasion surrounding individual myocytes. (E-F) Tumor invasion of the dermis. Arrows indicate normal epidermis, arrowheads indicate infiltrating tumor cells in dermis. (G,H) Tumor invasion of the epidermis eventually leads to loss of keratinocytes and severe skin ulceration in both genotypes. Arrows indicate intact epidermis at the border of the ulceration, arrowheads indicate crust formation, asterisks indicate the tumor.

Histological analysis of primary tumors and lung metastases of LLC

Primary tumors and spontaneous lung metastases from TAFI-deficient and wildtype mice were analyzed in more detail. No histological differences between tumors arisen in the presence or absence of TAFI were observed. The primary tumors showed all the histological characteristics of rapid growth. They existed of highly mitotic anaplastic cells having pleomorphic nuclei with conspicuous nucleoli and ample cytoplasm. Numerous normal as well as abnormal mitoses were observed independent of TAFI-genotype (Figure 4a,b). In larger nodules the central parts were necrotic. In addition, no differences were detected in the ability of tumor cells to invade skin tissue in the presence or absence of TAFI. All tumors diffusely invaded subcutaneous muscle tissue (Figure 4c,d), the dermal (Figure 4e,f) and epidermal layers of the skin, resulting in skin destruction and subsequent skin ulceration (Figure 4g,h). Detailed analyses of the lungs revealed the presence of both parenchymal and pleural metastases with cellular characteristics similar to the primary tumor (Fig 5a,b). Again, differently sized anaplastic cells with large vesicular nuclei containing multiple nucleoli and normal as well as abnormal mitotic figures could be identified in TAFI-deficient and wildtype mice (Fig 5c,d). However, the number of mitotic figures in these pulmonary nodules was less and the cells have an increased amount of cytoplasm when compared with the primary tumor. In addition, many tumor nodules had large necrotic areas associated with local hemorrhage (Fig 5a,b). Taken together, microscopic analyses of LLC tumors and lung metastases demonstrated that the absence of TAFI did not influence tumor architecture and invasive capacity.

Figure 5.



Histological analysis of lung metastases in TAFI-deficient (A,C) and wild-type (B,D) mice. Lungs were isolated 14 days after primary tumor resection, paraffin embedded, sectioned and stained with hematoxylin/eosin.
(A-D) Detailed analyses of the lungs revealed the presence of both parenchymal and pleural metastases composed of pleomorphic anaplastic cells having vesicular nuclei with multiple nucleoli and showing normal and abnormal mitotic figures (arrows) in TAFI-deficient and wildtype mice. Many tumor nodules had large necrotic areas associated with local hemorrhage (arrowheads).

Discussion

The findings presented here show that primary tumor growth and metastasis can successfully occur in the absence of TAFI. Moreover, our results indicate that primary and metastatic tumor growth is not affected by the absence of TAFI.

It is well established that components of the hemostatic system, including fibrin and plasminogen play a role in tumor development. Why doesn't TAFI-deficiency affect tumor growth? We tested our hypothesis that TAFI controls tumor development using B16-BL6 melanoma, T241 fibrosarcoma and Lewis lung carcinoma (LLC) cells because it has been established that the growth of these tumors is sensitive to coagulation and fibrinolytic protein deficiencies[51-54]. Subcutaneous growth of T241 fibrosarcoma is strongly reduced in mice lacking plasminogen[52]. In this study it was shown that plasminogen-deficient mice fail to suppress the accumulation of tumor-infiltrating macrophages, which is normally regulated by tumor cell-mediated plasminogen activation. However, the same study revealed that a combined deficiency of the plasminogen activators, uPA and tPA, or the absence of uPA alone just slightly decreased tumor growth in this model. Tumor growth was normal in mice lacking tPA or PAI-1. In contrast, using the same model, Gutierrez et. al.[51] have shown that primary tumor growth is critically dependent on uPA and PAI-1. In addition, they found that spontaneous metastasis of T241 fibrosarcoma cells to the lung and brain is almost completely abolished in the absence of uPA and PAI-1. Others have shown that hematogenous metastasis formation but not primary growth of LLC is reduced in PAI-1 overexpressing mice[55]. These effects were associated with increased expression of PAI-1 in the primary tumor and lungs, suggesting that proteolytic inhibition might explain the inhibition of metastasis. In another study, experimental pulmonary metastasis formation and primary growth of B16-BL6 melanoma cells in the hind limb of mice lacking PAI-1 was not affected[56]. Further studies in plasminogen-deficient mice by Degen's group revealed that plasminogen also contributes to the subcutaneous growth of LLC[54]. Plasminogen-deficiency reduced the growth of LLC and dissemination to regional lymph nodes and prolonged the survival after primary tumor resection. In addition, primary tumors were less ulcerating and less hemorrhagic in plasminogen-deficient mice compared to wildtype mice. Plasminogen-deficiency did not affect spontaneous pulmonary metastasis of LLC. Similar to T241 fibrosarcoma, LLC infiltration into adjacent skin tissues was unaffected in plasminogen-deficient mice. Moreover, the effects on subcutaneous tumor growth were small and have been contradicted in a recent publication from the same group where they reported that plasminogen does not determine the subcutaneous growth of LLC and T241 fibrosarcoma[57]. Thus, plasminogen-deficient mice are able to generate sufficient proteolytic activity for efficient tumor development and metastasis. However, in another anatomical location, the footpad, plasminogen-deficiency severely suppressed LLC and T241 fibrosarcoma tumor growth. Interestingly, tumor vessels in mice lacking plasminogen were occluded by microvascular thrombi that were absent in mice also deficient for fibrinogen. In addition, tumor growth was restored in mice lacking both plasminogen and fibrinogen[57]. Recent

experiments in fibrinogen-deficient mice and the use of a specific thrombin inhibitor have directly proven that fibrinogen and thrombus formation are essential for efficient metastatic seeding of circulating LLC and B16-BL6 tumor cells, either after intravenous injection or originating from a primary tumor[11;53].

We hypothesized that TAFI-deficiency would influence tumor development, for example through increased plasmin formation. Unbalanced plasmin formation inhibits tumor growth[58;59] and fibrinolytic therapy has been shown to potentially inhibit pulmonary seeding of intravenously injected mammary carcinoma cells in rats[60;61]. Conceivably, the absence of TAFI might increase fibrin degradation and premature thrombus removal, causing decreased experimental and spontaneous metastasis formation. Disturbance of tightly controlled plasminogen activation might cause decreased primary tumor growth and tumor cell migration resulting in the formation of less experimental and spontaneous metastasis[32;62]. On the other hand, increased plasminogen activation in the absence of TAFI could cause increased matrix degradation in the primary tumor resulting in increased tumor growth, efficient entrance of tumor cells into the circulation and elevated spontaneous metastasis formation. However, none of these possible effects were seen.

We suggest several potential explanations for the lack of differences in tumor growth and metastasis in TAFI-deficient mice. First, TAFI activation does not result in a significant change in plasmin formation that can influence the growth and metastasis formation in these cancer and metastasis models. For example, enhanced plasmin formation due to the absence of TAFI may not result in a significant increase in plasmin activity due to the presence of α2-antiplasmin that inhibits the additional plasmin. Alternatively, other proteins, including PAI-1 or other carboxypeptidases or mechanisms exist that may neutralize the effect of TAFI-deficiency on plasmin activation or activity. An alternative explanation for the lack of effect on tumor growth is that excess plasmin in TAFI-deficient mice has no effect at all in the models used. Similar observations were reported by others who studied TAFI in a pulmonary fibrinolysis model and in leukocyte migration[63], processes that are also activated in cancer. The inhibitory effects that accompanied TAFI-deficiency in these models were elucidated in and restricted to plasminogen-compromised mice. In other words, the plasminogen system may already be maximally involved in these processes. Similarly and as a consequence, tumor growth might have become insensitive to additional plasminogen activation. A second explanation for the lack of effect of TAFI-deficiency is that TAFI is not activated at all and does not regulate plasminogen activation in cancer and metastasis. Unfortunately, at present no tools are available to determine the presence of activated TAFI in mouse or human cancer tissues. We made an attempt to investigate the presence of activated TAFI in tumor-bearing mice by intravenous injection of a synthetic substrate for activated TAFI. Analysis of the amount of cleaved substrate in plasma isolated 10 minutes later, did not reveal a significant difference between TAFI-deficient and wildtype mice. Thus as of yet, we cannot draw a conclusion on the activation state of TAFI.

In conclusion, our findings show that TAFI is not needed for primary experimental tumor growth and metastasis formation.

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Chapter 4

Thrombin-activatable Fibrinolysis Inhibitor levels in prostate cancer patients

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Summary

Prostate cancer has historically been associated with hemostatic abnormalities. High plasma levels of markers of fibrinolysis indicate that the plasminogen activation system is activated in patients with prostate cancer. Thrombin-activatable fibrinolysis inhibitor (TAFI) is a B-type carboxypeptidase present in blood that can inhibit plasminogen activation. We hypothesized that TAFI may regulate activation of the fibrinolytic system in prostate cancer patients. This study was undertaken to investigate whether TAFI antigen and activity levels are influenced in patients with prostate cancer. We found that circulating plasma antigen and activity levels of TAFI are normal in prostate cancer patients and independent of the presence of metastases. Furthermore, TAFI levels were not associated with progression of the disease or with the presence of thrombosis. Our results suggest that TAFI does not play a major role during prostate cancer.

Introduction

It is well recognized that cancer patients have a high incidence of hemostatic complications such as disseminated intravascular coagulation[1], venous thromboembolism[2] and hemorrhage[3]. It has been reported that advanced prostate cancer is responsible for up to 25% of incidences of disseminated intravascular coagulation, presenting as a bleeding tendency with or without venous thrombosis[4]. There is also substantial evidence that the hemostatic system is involved in cancer progression and the formation of metastases. For example, proteins involved in maintaining hemostasis also regulate angiogenesis to support tumor growth. Clinical studies investigating the role of the hemostatic system in prostate cancer have indicated the presence of abnormal levels of proteins of the coagulation and fibrinolytic systems such as antithrombin III[5;6], prothrombin fragment 1 and 2[7], thrombin-antithrombin III (TAT) complexes[7;8], plasmin-α2-antiplasmin (PAP) complexes[8], plasminogen activator inhibitor (PAI-1)[6] and fibrin degradation products[7-9]. These abnormalities often reflect tumor malignancy and correlate with disease prognosis. The prevalence of these circulating markers is rather low in patients with primary cancer but high in patients with advanced prostate cancer[7;8;10]. Adamson and colleagues have shown that the levels of fibrin degradation products correlate with bone scan positivity in prostate cancer patients[9]. Thus, activation of the fibrinolytic system is seen in patients with prostate cancer.

Proteolytic enzymes involved in fibrinolysis are the serine proteases of the plasminogen activation system, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) and plasmin[11]. Both tPA and uPA generate plasmin by proteolytical cleavage of its zymogen plasminogen. Plasmin cleaves its substrates behind a lysine or arginine, which then become carboxy-terminal. Such carboxy-terminal basic amino acid residues in fibrin provide high affinity binding sites for plasminogen and facilitate efficient plasminogen activation by tPA and uPA[12-15].

Thrombin-activatable fibrinolysis inhibitor (TAFI), also known as carboxypeptidase U, R or plasma procarboxypeptidase B (EC 3.4.17.20) links the coagulation and fibrinolytic systems and regulates fibrinolysis *in vitro*[16] and *in vivo*[17-20]. When converted into its active form by thrombin[16], plasmin[21] or trypsin[22], TAFI is a carboxypeptidase B-type enzyme that specifically cleaves carboxy-terminal lysine or arginine residues from proteins. TAFI-mediated removal of these carboxy-terminal amino acids from fibrin attenuates efficient plasminogen activation into plasmin[23]. Thrombin-mediated activation of TAFI is stimulated 1250-fold by the endothelial cell receptor thrombomodulin[24]. Circulating TAFI antigen levels broadly vary between different healthy individuals and correlate with TAFI activity and clot lysis time[25;26]. Recently, several studies have been reported that indicate an association between elevated TAFI levels and thrombosis[27-31]. Reduced TAFI activity, but not TAFI antigen, in patients with acute promyelocytic leukemia was possibly due to proteolytic inactivation by excess fibrinolytic activity[32].

In the past, ε-aminocaproic acid (εACA), a lysine analogue that inhibits plasminogen activation, has been successfully used for the treatment of hemostatic complications in

prostate cancer patients[33]. This indicates that the plasminogen activation system and particularly carboxy-terminal lysines play an important role in these complications. We hypothesize that TAFI may influence cancer growth and/or hemostatic complications in patients with prostate cancer. Here we measured the plasma TAFI antigen and TAFI activity levels in patients with primary and metastasized prostate cancer.

Materials and methods

Patients and controls

We collected samples from 119 patients with prostate cancer. For controls, plasma samples were collected from 22 males with no evidence of cancer. Blood was collected by venepuncture in 3.2% (w/v) sodium citrate (1:10) (Venoject, Terumo Europe N.V. Leuven, Belgium). The samples were centrifuged for 15 minutes (2000g) and the supernatant was stored at –80^{o}C until use. In all samples from cancer patients routine prostate specific antigen (PSA) was determined.

Measurement of TAFI antigen and activity

TAFI antigen was determined with an enzyme-linked immunosorbent assay (ELISA) as has been described[34]. TAFI activity in plasma was measured as follows. Plasma was diluted 5 times in 50 mM Hepes, pH 7.4. Activation of TAFI was performed at room temperature by the addition of 5 µl of plasma to 50 µl activation buffer containing 50 mM Hepes, pH 7.4, 40 nM rabbit lung thrombomodulin (American Diagnostica, Greenwich, CT), 10 nM thrombin (Enzyme Research Laboratories, South Bend, IN), 20 mM $CaCl_2$ and 8 mM hippuryl-Arg (Bachem, Bubendorf, Switzerland), a substrate for activated TAFI. After 30 minutes, the reaction was stopped by 50 µl 1M HCl. After the addition of internal standard (20 µl of 22.5 µM O-methylhippuric acid) cleaved substrate was extracted with 300 µl ethylacetate, air-dried, dissolved in 150 µl 50 mM K_3PO_4, 20% acetonitril, pH 3.5 and analyzed using high performance liquid chromatography (HPLC; XTerra MS C18 3.5 µm, 4.6 x 100 mm column, Waters Chromatography BV, Ettenleur, The Netherlands). To distinguish between constitutive active carboxypeptidase (carboxypeptidase N) and active TAFI in plasma, carboxypeptidase activity sensitive to a potato carboxypeptidase inhibitor (CPI) was considered to be TAFI activity.

Results and discussion

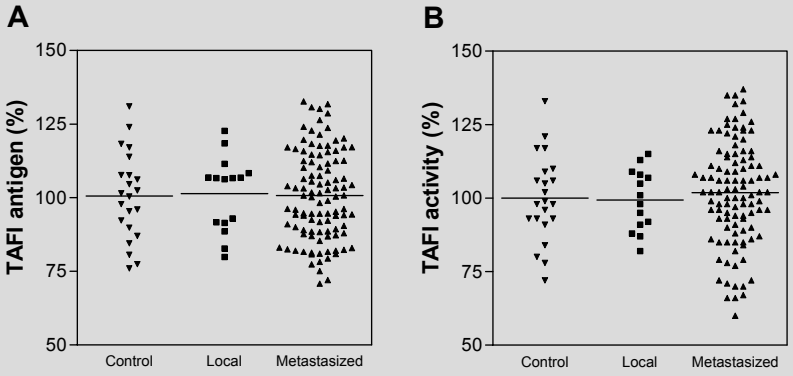
Patients

Hundred and nineteen prostate cancer patients and twenty-two age-matched controls without evidence of cancer were investigated. Hundred and four of the patients had metastases to distant organs and fifteen patients had cancer limited to the prostate. The mean age in the group of non-metastatic prostate cancer patients was 64 yr (range, 44 - 74 yr) and in the group of patients with metastases 67 yr, (range, 42 - 87 yr). The control population included patients with a mean age of 60 yr (range, 40 - 88 yr). The median serum PSA levels in the patients with prostate cancer were 154 ng/ml (range, 0 – 9513 ng/ml) in the group with metastases and 0.8 ng/ml (range, 0 – 30 ng/ml) in the non-metastatic patient group.

TAFI levels

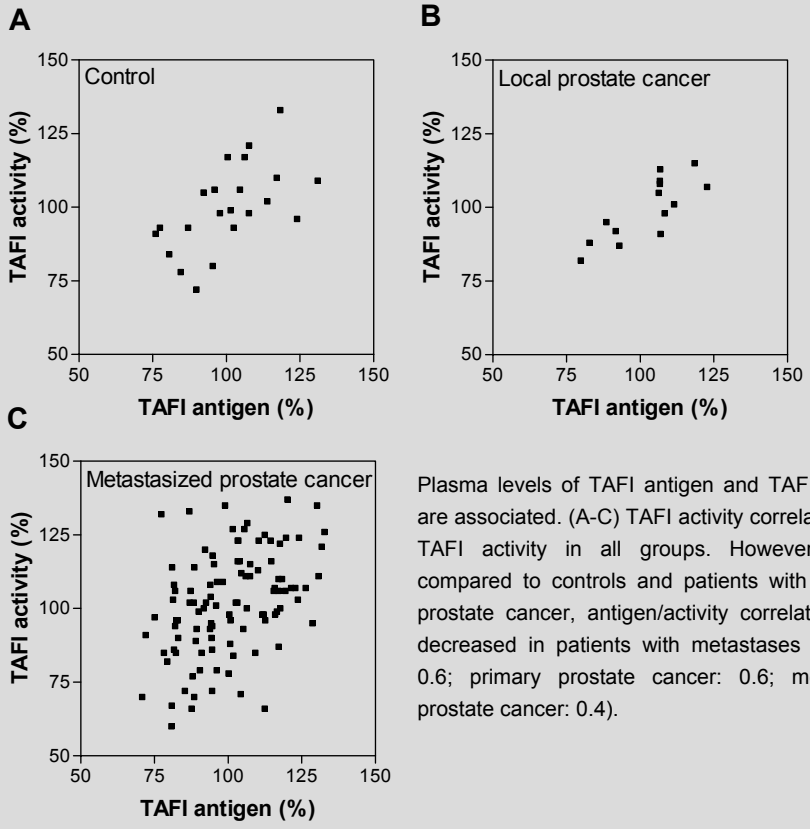
TAFI antigen and activity levels in the control group were normalized and considered 100%. When expressed as a percentage of that in controls (100 ± 3%, n=22), the mean TAFI antigen level in primary prostate cancer patients was 101 ± 3% (mean ± SEM, n=15) and 101 ± 1% (n=104) in metastasized prostate cancer patients (Figure 1a). Compared to controls (100 ± 3%, n=22), TAFI activity in plasma of primary prostate cancer patients was 99 ± 3% (mean ± SEM, n=14) and 102 ± 2% (n=104) in patients with metastasized prostate cancer (Figure 1b). These results indicate that TAFI levels did not change in prostate cancer patients. We hypothesized that TAFI may only be significantly affected in prostate cancer patients with high circulating levels of an indicator of prostate cancer activity, prostate specific antigen (PSA). However, TAFI antigen and activity did not correlate with PSA in patients with local tumor burden or metastases (not shown). In control subjects, TAFI antigen and activity will be highly correlated with each other. In other words: TAFI activity is fully determined by the amount of TAFI antigen. We investigated whether prostate cancer influences the relation between TAFI antigen and TAFI activity. We found that, although TAFI activity correlated with TAFI antigen in all groups, this correlation was strongly reduced in patients with metastases (Pearson correlation in control: 0.6; primary prostate cancer: 0.6; metastatic prostate cancer: 0.4) (Figure 2a-c). This observation could not be explained by disease progression (PSA levels). TAFI antigen or activity levels were not associated with the incidence of thrombosis.

Figure 1.



TAFI antigen and activity levels were measured in plasma of prostate cancer patients and controls. TAFI antigen and activity levels are expressed as percentage of control. TAFI antigen (A) and activity (B) levels are not different between patients with primary prostate cancer, metastasized prostate cancer and controls.

Figure 2.



Plasma levels of TAFI antigen and TAFI activity are associated. (A-C) TAFI activity correlated with TAFI activity in all groups. However, when compared to controls and patients with primary prostate cancer, antigen/activity correlation was decreased in patients with metastases (control: 0.6; primary prostate cancer: 0.6; metastatic prostate cancer: 0.4).

Discussion

Abnormalities of the coagulation and fibrinolytic systems are frequently found in cancer patients. Here we show that circulating plasma levels and activity of a regulator of fibrinolysis, TAFI, are normal in prostate cancer patients with or without evidence of metastases.

Our findings are somewhat unexpected. Based on the fact that the coagulation (thrombin) and/or fibrinolytic (plasmin) system are induced in prostate cancer patients, we expected that TAFI would be activated and consumed. Alternatively, TAFI levels could be negatively affected by inactivation of the enzyme through cleavage by increased plasmin present in prostate cancer patients. However, we did not observe abnormal TAFI antigen or activity levels in these patients. In the past, only one other similar study has been reported in patients with cancer. Although patients with acute promyelocytic leukemia (APL) had normal TAFI antigen levels, Meijers et al.[32] have shown that a large amount of TAFI circulated in a form that could not be activated. The authors suggest that hyperfibrinolysis is responsible for the decreased activity of TAFI in these patients. When compared to prostate cancer, APL is associated with an even higher activation state of the fibrinolytic system. Apparently, the relatively low amount of activated plasminogen in prostate cancer patients, compared to patients with APL, does not affect TAFI.

Taken together, the growth and metastasis of prostate cancer and the hemostatic complications that are often observed in prostate cancer patients are not associated with changes in TAFI. Moreover, prostate cancer does not seem to affect the activation of circulating TAFI.

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Chapter 5

Amyloid endostatin induces endothelial cell detachment by stimulation of the plasminogen activation system

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Summary

Endostatin is a fragment of collagen XVIII that acts as an inhibitor of tumor angiogenesis and tumor growth. Antitumor effects have been described using both soluble and insoluble recombinant endostatin. However, differences in endostatin structure are likely to cause differences in bioactivity. In the present study we have investigated the cellular effects of insoluble endostatin. We previously found that insoluble endostatin shows all the hallmarks of amyloid aggregates and potently stimulates tPA-mediated formation of the serine protease plasmin. We here show that amyloid endostatin induces plasminogen activation by endothelial cells, resulting in vitronectin degradation and plasmin-dependent endothelial cell detachment. Endostatin-mediated stimulation of plasminogen activation, vitronectin degradation and endothelial cell detachment is inhibited by carboxypeptidase B indicating an essential role for carboxy-terminal lysines. Our results suggest that amyloid endostatin may inhibit angiogenesis and tumor growth by stimulating the fibrinolytic system.

Introduction

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is important during various pathological processes, including inflammation and tumor growth. Antiangiogenic therapy is being considered as a potentially powerful new therapy for cancer and other angiogenesis-dependent diseases. Endostatin is one of the most potent inhibitors of angiogenesis and can induce tumor regression in mice[1]. Clinical trials are currently ongoing[2;3]. Originally, endostatin was purified from conditioned medium of murine hemangioendothelioma (EOMA) cells as a proteolytically cleaved fragment of collagen XVIII. Generation of endostatin can be achieved by cleavage of collagen by cathepsin L[4], matrilysin[5] or elastase[6].

Endostatin has distinct antiangiogenic and Antitumor activities in several animal models[7]. Tumor growth was significantly inhibited by intraperitoneal or subcutaneous injection of endostatin without induction of acquired drug resistance[1;8]. In addition, rat endostatin induced significant inhibition of carcinogen induced mammary tumor growth[9]. Endostatin is also bioactive using delivery approaches such as DNA vaccination and viral expression[10;11]. However, contrary to this, no antiangiogenic effect of endostatin was seen in other studies despite high serum levels[12-15]. This paradox has lead to discussions about the efficacy of endostatin[16]. Until now, this paradox remains unsolved. Possibly, the outcome of endostatin treatment will depend on its structure (see below) and/or the presence of factors provided at the site of action.

A variety of molecular mechanisms have been proposed to underlie endostatin activity. Endostatin blocks vascular endothelial growth factor mediated signalling through KDR/Flk-1[17] and induces endothelial cell apoptosis, associated with decreased levels of anti-apoptotic proteins Bcl-2 and $BclX_L$[18]. Endostatin can bind tropomyosin, an actin stabilizing protein, and has been suggested to disrupt microfilament integrity ultimately causing endothelial cell apoptosis[19]. Direct effects of endostatin on cell adhesion may be caused by suppression of integrin function[20;21]. Other studies have implicated that endostatin acts on the proteolytic system by binding and inhibiting active metalloproteinase (MMP)-2[22;23] or down regulating the urokinase plasminogen activator system[24]. However, it is not clear to what extent these activities contribute to the antiangiogenic and antitumor effects of endostatin *in vivo*.

A complicating factor is that endostatin is used in a soluble, as well in an insoluble form. Whereas both forms have been reported to inhibit tumor growth, the regression of tumors has only been reported with insoluble endostatin[1]. Different structural forms of endostatin have distinct bioactivities and may therefore produce their antiangiogenic effects through distinct mechanisms. The structure of soluble, globular endostatin has been elucidated[25;26]. Recently we found that endostatin is a protein with high propensity to form amyloid fibers through extensive cross-β sheet formation[27;28]. Moreover, we established that only insoluble endostatin but not soluble endostatin stimulates tPA-mediated plasminogen activation and induces cell toxicity[27;28]. Here we have studied the effect of insoluble endostatin, to which we

refer as amyloid endostatin, on endothelial cell-mediated plasmin formation and cell adhesion.

Materials and methods

Proteins and reagents

The cDNA for murine endostatin (kindly provided by dr. Fukai, Boston) was amplified by PCR and cloned into the prokaryotic expression vector pET15b (Novagen, WI). Recombinant murine endostatin was produced by Escherichia *coli* as described[1] and resuspended in phosphate-buffered saline (PBS). Soluble Pichia *pastoris*-produced endostatin was provided by Entremed, Inc. (Rockville, Maryland). Plasminogen was from Sigma® Chemical Co (St. Louis, MO) or purified from plasma as described[29], tPA and plasmin substrate S-2251 were from Chromogenix AB (Mölndal, Sweden), pefabloc® tPA was from Pentapharm AG (Switzerland), porcine pancreas carboxypeptidase B was from Boehringer Mannheim® (Mannheim, Germany), potato carboxypeptidase inhibitor and rabbit polyclonal antibody against vitronectin were from Calbiochem® (La Jolla, CA). Anti-plasminogen monoclonal antibody and anti-tPA polyclonal antibodies were from American Diagnostica (Greenwich, CT).

Preparation of yeast-produced amyloid endostatin

Pichia *pastoris*-produced endostatin (Entremed, Inc. Rockville, Maryland) was denatured by dialyses against 8 M urea, 10 mM Tris (pH 7.5) and 10 mM β-mercaptoethanol. The denaturing buffer was subsequently removed by extensive dialysis against H_2O. Amyloid endostatin became visible as a white precipitate.

Binding experiments

Binding of plasminogen and tPA was carried out in 96-well microtiter plates coated overnight at room temperature with 50 µl amyloid endostatin (20 µg/ml) or fibrin fragments (DESAFIB-X®, Chromogenix, 20 µg/ml) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02 % NaN_3, pH 9.6). The wells were blocked with 3% bovine serum albumin (BSA) in PBS for one hour and washed three times with PBS containing 0.3% BSA (PBS/BSA). Where indicated the wells were treated with porcine pancreas carboxypeptidase B (50 µg/ml) for one hour at 37 °C in PBS containing 3% BSA and washed 3 times with PBS/BSA. Plasminogen and tPA, at various concentrations, were allowed to bind for one hour. After 3 washes, bound plasminogen or tPA were detected with specific anti-plasminogen and anti-tPA antibodies followed by peroxidase conjugated secondary antibodies. Peroxidase activity was measured using ortho-phenylenediamine as substrate. The reaction was stopped by the addition of 1 M H_2SO_4 and absorbance was measured at 490 nm.

Measurement of plasmin activity

The reactions were performed at 37°C in HBS buffer (20 mM Hepes, 4 mM KCl, 137 mM NaCl, 3 mM $CaCl_2$, 0.1 % BSA, pH 7.4) containing 50 µg/ml of plasminogen with various concentrations of amyloid endostatin, DESAFIB®-X or with a control sample. The reactions were started by the addition of tPA at a final concentration of 30 U/ml. At several time points, 20 µl samples were taken and the reaction was stopped with 20 µl buffer containing 150 mM ε-aminocaproic acid (εACA) and 150 mM EDTA. Plasmin activity was determined in 96-well plates after the addition of 20 µl chromogenic substrate S-2251 at a final concentration of 1.6 mM. Increase in absorbance was measured at 405 nm for 10 min. When applicable test samples were preincubated for 30 minutes with 25 µg/ml carboxypeptidase B and/or 50 µg/ml potato carboxypeptidase inhibitor.

Plasminogen activation by endothelial cells was determined by SDS-PAGE analysis and western blotting. Conditioned medium was concentrated five times with nanosep 10K Omega (Pall Life Science, Portsmouth, UK). Plasmin activity was analyzed by adding 10 µl of concentrated conditioned medium to S-2251 (1.25 mM) and subsequent reading at 405 nm.

Cell culture

Bovine pulmonary arterial endothelial cells (BPAEC, CCL-209) were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle Medium (Gibco BRL, Invitrogen Corporation, UK) with the supplement of 10% FCS and antibiotics. BPAEC were seeded onto 48-well culture plates (Costar Inc. NY) and grown to confluency in culture medium. The cells were washed two times with PBS and incubated with yeast-produced amyloid endostatin in Human Endothelial-SFM Basal Growth Medium (Gibco BRL, Invitrogen Corporation, UK) supplemented with antibiotics.

Assay of endothelial cell detachment

BPAEC were incubated with amyloid endostatin in the presence or absence of plasminogen. Were indicated, 50 µg/ml carboxypeptidase B was used for complete blockage of plasminogen activation and 20 µM Pefabloc® tPA was used to inhibit tPA activity. After 24 hours the cells were photographed using phase contrast microscopy. The supernatant was stored for further analyses. Cell detachment was analyzed by encircling the non-cell area in four different photographs using Adobe® Photoshop® version 6.0 (Adobe Systems Inc., CA, USA) and subsequently quantified by calculating the percentual coverage of the selected area using Optimas 6.0 software (DVS, Breda, The Netherlands).

Tumor experiments

Male 6 to 8 week old BALB/c mice (General Animal Laboratory, University Medical Center Utrecht, Utrecht, The Netherlands) were used. All mice were fed a diet of animal chow and water *ad libitum*. Experiments were performed according to the guidelines of the Utrecht Animal Experimental Committee, University Medical Center (Utrecht, The Netherlands).

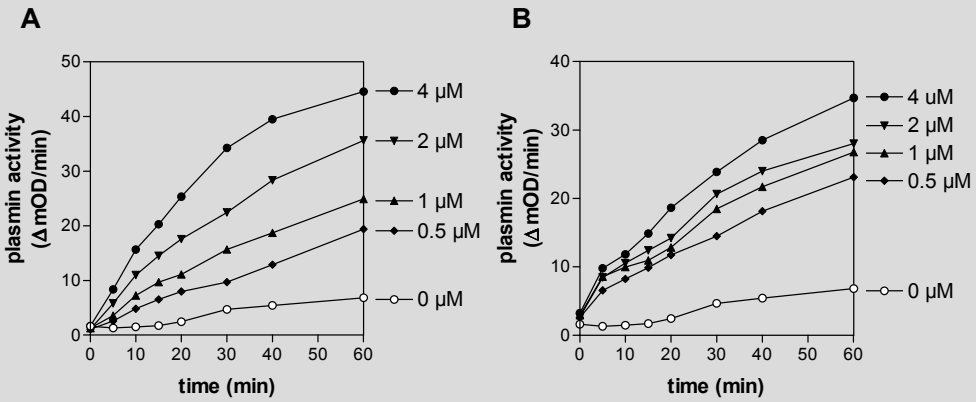
Mice were inoculated subcutaneously with $1x10^6$ C26 colon carcinoma cells in 200 µl PBS. Escherichia *coli*-derived amyloid endostatin (20 mg/kg/day) or control solvent was given by daily subcutaneous injection, starting just after tumor cell injection and stopped at the end of the experiment. This results in this model in approximately 50% inhibition of tumor growth. Carboxypeptidase B was given continuously using a mini-osmotic pump (Alzet® pump, Alza, Palo Alto, California, USA, type 2002 [14 days]) containing 200 µl carboxypeptidase B at a concentration of 5 mg/ml. The pump was implanted subcutaneously in the dorsal skin fold. In mice bearing subcutaneous tumors, absence of contact between tumor deposit and pump was assured by implanting the pump in the contralateral side. Animals received an initial subcutaneous bolus injection of 40 µg carboxypeptidase B in 100 µl PBS at the time of tumor cell injection. Tumor diameters were determined on day 13 by calliper and tumor volume was calculated with the formula $width^2$ x length x 0.52. Significance of differences in tumor growth among groups was determined by the unpaired Students t test. $P < 0.05$ was considered to be statistically significant. Values represent mean number ± SEM.

Results

Amyloid endostatin stimulates plasmin formation

We previously showed that denatured endostatin forms amyloid fibrils and stimulates tPA-mediated plasminogen activation *in vitro*[27]. We show here that amyloid endostatin stimulates tPA-mediated plasminogen activation in a dose dependent manner (Figure 1a). Stimulation of tPA-mediated activation of plasminogen by amyloid endostatin was as potent as stimulation by fibrin fragments, the classical stimulator of tPA-mediated plasminogen activation (Figure 1b). No activation of plasminogen was observed in the absence of tPA and amyloid endostatin alone did not convert the chromogenic substrate (not shown).

Figure 1.

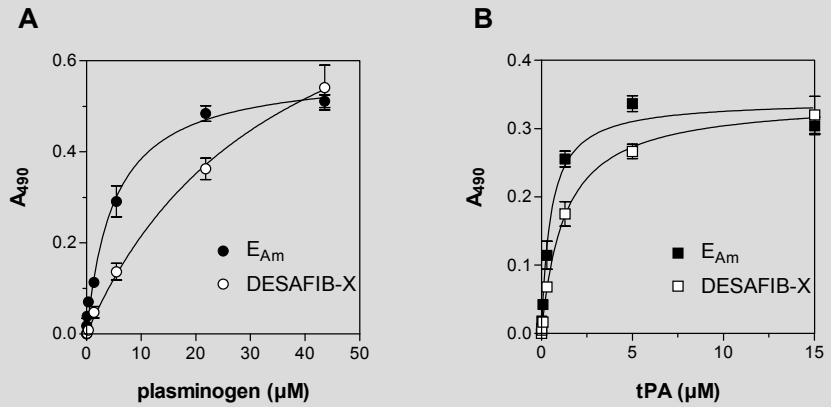


Amyloid endostatin-stimulated tPA-mediated plasminogen activation is concentration dependent. Different concentrations of amyloid endostatin (A) and fibrin fragments (B) were added to plasminogen. Plasminogen activation was started by addition of tPA. At the indicated time points samples were taken and plasmin activity was analyzed using the chromogenic substrate S-2251. These figures show a representative experiment out of five independent experiments.

Amyloid endostatin binds plasminogen and tPA

Because endostatin is a stimulator of plasminogen activation by tPA, we tested whether amyloid endostatin was able to bind plasminogen and tPA. Both plasminogen and tPA bound to immobilized amyloid endostatin in a dose-dependent and saturable manner (Figure 2a). Binding of tPA to amyloid endostatin (apparent dissociation constant (K_d) = 1.6 ± 0.3 nM) was similar to the binding of tPA to fibrin degradation products (K_d = 2.3 ± 0.4 nM) (Fig 2b). The affinity of plasminogen for amyloid endostatin (K_d = 2.7 ± 0.6 nM) was about 10 times higher than the affinity of plasminogen for fibrin fragments (K_d = 28.4 ± 5.8 nM).

Figure 2.



Amyloid endostatin binds to plasminogen and tPA. Binding of plasminogen (A) and tPA (B) to immobilized amyloid endostatin or fibrin fragments was measured by ELISA. Binding was detected using specific antibodies against plasminogen or tPA followed by peroxidase-conjugated secondary antibodies and substrate addition.

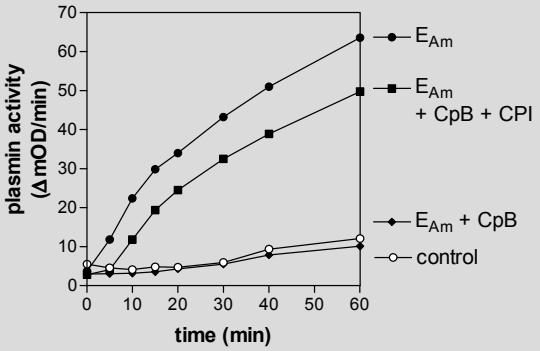
Carboxypeptidase B inhibits the tPA-stimulatory activity of amyloid endostatin

The binding of plasminogen and tPA to fibrin is mediated by the lysine-binding sites in the kringle domains of plasminogen and tPA and by carboxy-terminal lysine residues that are generated in fibrin during plasmin digestion[30]. Therefore, we investigated the importance of carboxy-terminal lysines in amyloid endostatin-mediated plasmin formation. To this end we used the porcine pancreas carboxypeptidase B (CpB) that removes basic (arginine and lysine) amino acids from proteins. Treatment with CpB reduced amyloid endostatin-stimulated plasminogen activation (Figure 3). The inhibitory effect of CpB on amyloid endostatin was greatly diminished by the addition of a specific carboxypeptidase inhibitor (CPI), indicating that the effect of CpB is due to carboxypeptidase activity. Binding analyses revealed that CpB treatment of amyloid endostatin abrogated plasminogen binding for more

than 90%, but had little or no effect on tPA binding. In line with this, plasminogen binding to amyloid endostatin was blocked by the lysine analogue ε-aminocaproic acid (not shown). These results clearly show that carboxy-terminal lysines are essential for efficient tPA-mediated plasminogen activation.

Figure 3.

Carboxypeptidase B blocks amyloid endostatin-induced tPA-mediated plasminogen activation. The formation of plasmin was induced by 4 μM amyloid endostatin. Prior to the start of the reactions with tPA, carboxypeptidase B was added for 30 minutes at 37°C. Carboxypeptidase inhibitor (CPI) was included to demonstrate that the effect of carboxypeptidase B was the result of specific activity. The figure shows a representative experiment out of five independent experiments.



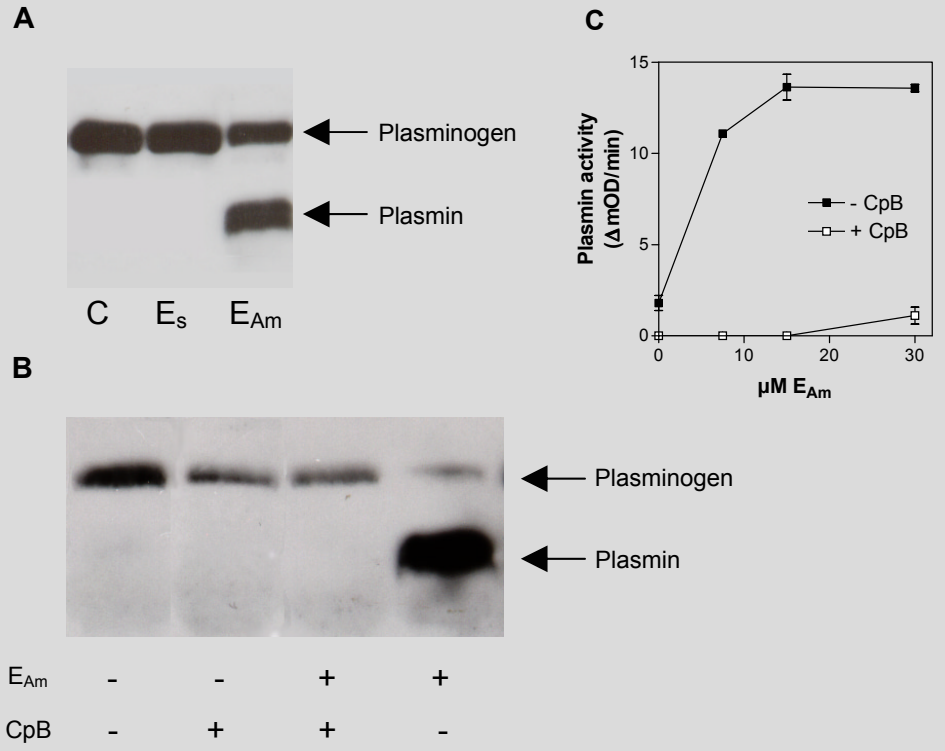
Amyloid endostatin stimulates plasminogen activation by endothelial cells

We next determined whether amyloid endostatin can stimulate plasminogen activation mediated by endothelial cells, a biological source of tissue-type plasminogen activator. Different concentrations of amyloid endostatin were added to confluent monolayers of endothelial cells in the presence of 0.4 μM plasminogen. After 24 hours, the medium was analyzed by western blotting. Plasminogen cleavage by plasminogen activator results in the formation of plasmin, which migrates at approximately 50 kD in gel electrophoresis. In contrast to soluble endostatin, amyloid endostatin treatment significantly enhanced cell-mediated plasmin formation (Figure 4a). Cells treated without amyloid endostatin did not convert plasminogen into plasmin. Also in endothelial cell-mediated plasminogen activation induced by amyloid endostatin, we found a similar dependence of amyloid endostatin activity on the presence of carboxy-terminal lysines. Addition of 50 μg/ml carboxypeptidase B totally abolished plasminogen activation (Figure 4b). Determination of chromogenic substrate conversion by concentrated medium indicated that plasmin was active (Figure 4c).

Amyloid endostatin causes endothelial cell detachment and extracellular matrix degradation

Sofar, we have shown that amyloid endostatin stimulates plasminogen activation both *in vitro* and on endothelial cells. Increased plasmin formation has been implicated in endothelial cell detachment[31-34]. Therefore, we studied the effects of amyloid endostatin on endothelial cell adhesion in the absence and presence of different plasminogen concentrations (Fig 5). While amyloid endostatin or plasminogen alone did not induce cell

Figure 4.

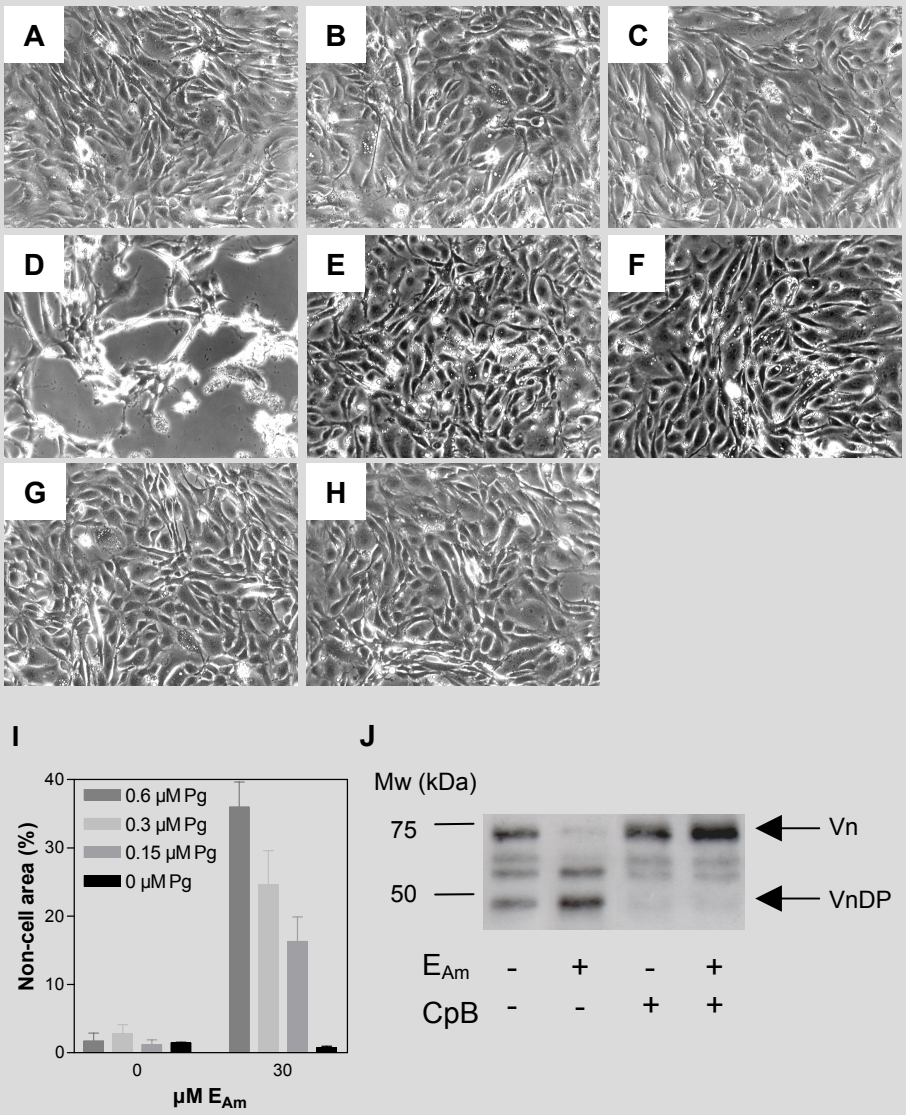


Amyloid endostatin stimulates endothelial cell mediated plasminogen activation. Plasmin formation in culture medium of control cells (C) and cells treated with 30 μM of amyloid endostatin (E_{Am}) or 30 μM of soluble endostatin (E_s) was analyzed by SDS-PAGE and western blotting using anti-human plasminogen antibody. (B) Addition of carboxypeptidase B (CpB) completely blocked amyloid endostatin-induced plasmin formation. (C) Plasmin activity was determined in culture medium of amyloid endostatin-treated cells using chromogenic substrate S-2251.

detachment (Figure 5a-c) co-treatment of the endothelial cells with plasminogen resulted in dramatic contraction of the cell bodies and complete rounding up of the cells (Figure 5d). Addition of carboxypeptidase B inhibited amyloid endostatin-mediated plasmin formation (Fig 4b) and subsequent cell detachment (Figure 5e). To asses the involvement of tPA in endostatin-induced plasminogen activation and cell detachment, 20 μM Pefabloc® tPA was used to inhibit tPA activity. This tPA inhibiting peptide completely inhibited plasmin-mediated cell detachment indicating an essential role for tPA in these processes (Figure 5f). Treatment with soluble endostatin alone (Figure 5g) or in combination with plasminogen (Figure 5h) had no effects.

The observed detachment of endothelial cells could be mediated by extracellular matrix degradation. Vitronectin (serum spreading factor) is an important component of the extracellular matrix involved in cell attachment and cell spreading. Vitronectin can be

Figure 5.



Amyloid endostatin causes endothelial detachment. Bovine pulmonary arterial endothelial cells were cultured to confluency and incubated with 30 µM of amyloid endostatin (E_{Am}) in the presence of different concentrations of plasminogen (Pg). After 24 hours the cells were photographed. (A) Control-treated endothelial cells. (B) 30 µM E_{Am} or (C) 0.6 µM plasminogen did not induce endothelial cell detachment. (D) Cell contraction and rounding up induced by 30 µM E_{Am} and 0.6 µM plasminogen co-treatment. Addition of (E) 50 µg/ml carboxypeptidase B or (F) 20 µM Pefabloc® tPA completely blocked E_{Am}-induced endothelial cell detachment. Soluble endostatin in the absence (G) or presence (H) of plasminogen had no effects. (I) Cell detachment was quantified as described in the material and methods section. Values represent mean number ± SEM. (J) Vitronectin (Vn) degradation was analyzed by western blotting. VnDP, Vn degradation product.

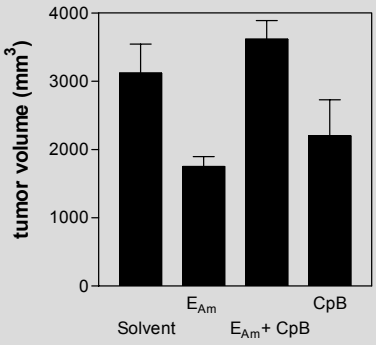
degraded by plasmin[35]. Vitronectin degradation was studied in lysates of amyloid endostatin-treated endothelial cells by western blotting. Whereas only small amounts of vitronectin degradation products were generated in the presence of plasminogen alone, co-treatment with amyloid endostatin potently increased vitronectin degradation (Fig 5j). Carboxypeptidase B, which blocked plasmin formation (Figure 4b), also abrogated vitronectin degradation.

Inhibitory activity of amyloid endostatin on subcutaneous tumor growth is reverted by carboxypeptidase B

To determine the possible role for carboxy-terminal lysine residues in the antitumor effect of amyloid endostatin *in vivo* we treated mice with amyloid endostatin in the absence or presence of CpB. Treatment was started directly after tumor cell injection and stopped on day 13. Amyloid endostatin-treated mice showed approximately 50% reduced growth of a subcutaneous colon carcinoma (P = 0.046 treatment *versus* control), while the presence of carboxypeptidase B completely abolished this reduction (Figure 6). The antitumor effect of CpB alone was not significant (P = 0.229 CpB *versus* control).

Figure 6.

Inhibition of tumor growth by amyloid endostatin is abolished by carboxypeptidase B treatment. Mice (n=4/group) were inoculated subcutaneously with $1x10^6$ C26 colon carcinoma cells and treated daily with amyloid endostatin or control solvent in the presence or absence of carboxypeptidase B as indicated. The inhibitory effect of amyloid endostatin on tumor growth was completely blocked by continuous administration of carboxypeptidase B. The experiment shown is a representative of three independent experiments.



Discussion

The formation of plasmin by activation of its zymogen plasminogen is associated with degradation of the extracellular matrix as it occurs in the dissolution of blood clots, tissue remodeling, invasive growth of cancer cells and angiogenesis[36-39]. Plasmin mediates proteolysis of the extracellular matrix by degrading fibrin and other matrix molecules. In addition, plasmin mediates proteolysis indirectly by the activation of metalloproteinases, which further degrade the extracellular matrix. During angiogenesis, extracellular matrix components regulate cell proliferation, migration and survival 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 $\alpha_v\beta_5$[40]. Plasmin has been implicated as an important modulator of the interactions between cells and the extracellular matrix[32;41]. Plasmin may also liberate cytokines, such as TGFβ[42], from inactive precursors and could play a role in the formation of plasminogen breakdown products with antiangiogenic properties, including angiostatin and kringle 5[43]. Thus plasmin is a key enzyme in the angiogenic process.

Here, we have demonstrated that amyloid endostatin, a cofactor for tPA-mediated plasminogen activation, induces endothelial cell-mediated plasmin formation resulting in vitronectin degradation, cell remodeling and detachment.

Numerous studies revealed high expression of tPA in several human tumors[44-48]. Therapeutic administration of amyloid endostatin may result in stimulation of tPA activity that is produced in the tumor. In light of this, it is interesting to note that tPA expression by endothelial cells is induced by angiogenic factors, including basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF)[49]. Overstimulation of the plasminogen activation system may result in excessive matrix degradation. Induction of plasminogen activation has lead to endothelial cell detachment[31], inhibition of cell adhesion[32], endothelial cell destruction[33] or regression of capillary tubes[34]. Vitronectin plays an important role in endothelial cell survival[50]. We found that vitronectin, a main adhesive matrix protein present in serum and deposited in the extracellular matrix of cultured endothelial cells is degraded upon treatment with amyloid endostatin. Its breakdown may cause cell detachment and subsequent apoptosis.

With the discovery of tPA as a general cross-β sheet-binding protein[27] we have identified a molecule that may contribute to the cellular effects induced by amyloid proteins like endostatin. Indeed, a specific inhibitor of tPA activity completely blocked plasmin-mediated changes of endothelial cell morphology induced by amyloid endostatin.

We noted that several other antiangiogenic peptides, like amyloid endostatin can stimulate tPA-mediated plasminogen activation. Stimulatory activity towards plasminogen activation and antiangiogenic activity has been described for a cleaved or denatured conformation of antithrombin, aaATIII[51;52], prothrombin fragments[53;54], thrombospondin[55-58], maspin[59;60] and amphoterin[61;62]. This could implicate that a common antiangiogenic pathway may exist that is induced by tPA binding proteins (see also chapter 9). Clinical studies underscore that high tPA levels are associated with good prognosis in cancer patients[63-65].

In fibrin, the generation of carboxy-terminal lysines is key to the efficient activation of plasminogen, as they form high affinity binding sites for plasminogen and, less so, tPA. The importance of carboxy-terminal lysines in endostatin-mediated plasminogen activation and subsequent detachment of endothelial cells was shown since addition of carboxypeptidase B totally abrogated endostatin activity. Physiologically, plasminogen activation induced by partially degraded fibrin is regulated by thrombin-activatable fibrinolysis inhibitor (TAFI), a carboxypeptidase B-type enzyme present in blood[66;67]. We found that stimulation of tPA-

mediated plasminogen activation by endostatin was similarly inhibited by activated TAFI (not shown). Thus, TAFI could function as a regulator of the antitumor activity of endostatin.

In line with our *in vitro* data, co-treatment of mice with endostatin and carboxypeptidase B abolished the inhibitory effect of endostatin. We can not exclude that other carboxypeptidase B sensitive pathways involved in endostatin bioactivity may be affected, but these results strongly suggest that the plasminogen activation system plays an important role in endostatin function.

Our suggestion that increased plasminogen activation might be responsible for at least part of the endostatin effect on tumor growth is supported by data of others. Tumor vascularization and tumor invasion is prevented in the absence of PAI-1 when increased levels of plasmin are likely to be formed[68;69]. In addition, high concentrations of tPA are generated and PAP levels are elevated in patients with peripheral tumors that regress in response to tumor necrosis factor alpha (TNFα)[70;71].

Taken together, overstimulation of tPA by agents such as amyloid endostatin may result in excessive matrix degradation thereby preventing angiogenesis and tumor growth[72]. This is a novel pathway to intervene in tumor growth and warrants further study.

Acknowledgements

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Chapter 6

Recombinant endostatin forms amyloid fibrils that bind and are cytotoxic to murine neuroblastoma cells *in vitro*

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Summary

Endostatin is a fragment of collagen XVIII that acts as an endogenous inhibitor of angiogenesis and tumor growth. Antitumor effects have been described using both soluble and insoluble recombinant endostatin. However, differences in endostatin structure are likely to cause differences in bioactivity. In the present study we have investigated the structure and cellular effects of insoluble endostatin. We found that insoluble endostatin shows all the hallmarks of amyloid aggregates. Firstly, it binds Congo red and shows the characteristic apple-green birefringence when examined under polarized light. Secondly, electron microscopy shows that endostatin forms short unbranched fibrils. Thirdly, X-ray analysis shows the abundant presence of cross-β sheets, the tertiary structure that underlies fibrillogenesis. None of these properties were observed when examining soluble endostatin. Soluble endostatin can be triggered to form cross-β sheets following denaturation indicating that endostatin is a protein fragment with an inherent propensity to form amyloid deposits. Like β-amyloid, found in the brains of patients with Alzheimer's disease, amyloid endostatin binds to and is toxic to neuronal cells, whereas soluble endostatin has no effect on cell viability. Our results demonstrate a previously unrecognized functional difference between soluble and insoluble endostatin, only the latter acting as a cytotoxic amyloid substance.

Introduction

In recent years cancer therapy has seen the development of a new class of drugs: inhibitors of the formation of new blood vessels ('angiogenesis') which thereby limit blood supply to tumors. A number of antiangiogenic compounds have proven to be effective in eradicating tumors in mouse models and are now being further tested in clinical trials (reviewed in[1-4]). Endostatin, a naturally occurring fragment of collagen XVIII, is considered to be one of the most effective inhibitors of angiogenesis[3]. Early studies with bacterially produced (insoluble) endostatin have shown its potent inhibitory effect on tumor growth[5;6]. Although soluble endostatin (produced in yeast) can also inhibit tumor growth, permanent tumor regression has only been reported when using insoluble endostatin[3]. Effects of endostatin on endothelial cell proliferation[6], apoptosis[7-9] and migration[10;11] have been described, but it is not clear to what extent these activities contribute to the antiangiogenic and antitumor effects of endostatin *in vivo*. Moreover, it remains unclear why endostatin therapy works so well in some experiments[3] but remains ineffective in others[12-15] (and our own unpublished data). Different structural forms of endostatin are likely to have distinct bioactivities. Soluble and insoluble endostatin may therefore produce their antiangiogenic effects through distinct mechanisms. In the present report we set out to investigate the structural and biological differences between soluble and insoluble endostatin. Whereas the structure of soluble, globular endostatin has been elucidated[16;17], the structure of insoluble endostatin has not. Insoluble proteins may occur as amorphous aggregates but they may also occur as highly ordered 'amyloid' deposits[18]. In the latter case, the polypeptide backbones are in a β-sheet conformation and are stacked through intermolecular (rather than intramolecular) hydrogen bonds, thus forming a 'cross-β sheet'[19]. We found that endostatin is a protein with high propensity to form amyloid fibers through extensive cross-β sheet formation. Fibrillar endostatin binds to neuronal cells and causes neuronal cell death whereas soluble endostatin does not. Our results suggest that endostatin induces apoptosis as a result of its amyloid structure.

Materials and methods

Preparation of recombinant human endostatin from bacteria

Endostatin was purified from bacteria essentially as described[5]. In short, BL21(DE3) bacteria expressing endostatin were lysed in a buffer containing 8 M urea, 10 mM Tris (pH 8.0), 10 mM imidazole and 10 mM β-mercaptoethanol. Following purification over Ni-agarose, the protein sample was extensively dialyzed against H_2O. During dialysis endostatin precipitates as a fine white solid. Aliquots of this material were either stored at –80^{o}C for later use, or were freeze-dried prior to storage.

Recombinant human endostatin from yeast

Endostatin produced by the yeast strain Pichia *pastoris* was kindly provided by Dr. Kim Lee Sim (EntreMed, Inc., Rockville, MA, USA).

Preparation of aggregated yeast-produced endostatin

Soluble yeast endostatin was dialyzed overnight in 8 M urea and subsequently three times against H_2O. Like bacterial endostatin, yeast endostatin precipitates as a fine white solid.

Amyloid β (Aβ) and human islet amyloid polypeptide (hIAPP)

$A\beta_{1-40}$ (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) and hIAPP (KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY) were obtained from the peptide synthesis facility at the Netherlands Cancer Institute (Amsterdam, the Netherlands). The freeze-dried peptides were resuspended in phosphate-buffered saline (PBS) and were allowed to form cross-β structure over a period of three weeks at room temperature. Cross-β sheet formation was followed by Congo Red binding and examination of green birefringence under polarized light.

Congo Red staining

Freeze-dried bacterial endostatin was resuspended in either 0.1% formic acid or in dimethylsulfoxide (DMSO) and taken up in a glass capillary. The solvent was allowed to evaporate and the resulting endostatin material was stained with Congo Red (Sigma) according to the manufacturer's protocol.

Transmission electron microscopy (TEM)

Endostatin samples were applied to 400 mesh specimen grids covered with carbon-coated collodion films. After 5 min. the drops were removed with filter paper and the preparations were stained with 1% methylcellulose and 1% uranyl acetate. After washing in H_2O the samples were dehydrated in a graded series of EtOH and hexamethyldisilazane. Transmission electron micrographs were recorded at 60 kV using a JEOL-1 electron microscope.

X-ray diffraction analysis

Aggregated endostatin was solubilized in 0.1% formic acid and was taken up in a glass capillary. The solvent was then allowed to evaporate over a period of several days. Capillaries containing the dried samples were placed on a Nonius κCCD diffractometer. Scattering was measured using sealed tube MoKα radiation with a graphite monochromator on the CCD area detector for a period of 16 hours. Scattering from air and the glass capillary wall were subtracted using in-house software (VIEW/EVAL).

N1E-115 cell culture and differentiation

N1E-115 mouse neuroblastoma cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS), supplemented with antibiotics. Cells were differentiated into post-mitotic neurons by culturing them in DMEM containing 0.5% FCS, 1 mM cAMP and 1% DMSO for 48 hours.

Fluorescein isothiocyanate (FITC)-labeling and fluorescence microscopy

Soluble (yeast-produced) endostatin was dialyzed against 0.01 M $Na_2B_4O_7$, 0.15 M NaCl (pH 9.5) overnight. FITC was dissolved (1 mg/ml) in 0.1 M Na_2CO_3 (pH 9.5) and was added to the endostatin, Aβ or hIAPP solutions at 2 µg FITC/mg protein. After 4 hour incubation at room temperature free FITC molecules were removed by dialysis against 1 M Tris pH 7.5, and subsequently, against PBS. During this procedure endostatin stays soluble and becomes highly fluorescent. Aggregated FITC-labeled endostatin was prepared from soluble FITC-labeled endostatin as above. Freshly solubilized as well as pre-aggregated Aβ and hIAPP were labeled with FITC by the same protocol. Cells grown on glass coverslips were exposed to FITC-labeled soluble or aggregated endostatin (5 µM), freshly resuspended or pre-aggregated Aβ or hIAPP for 6 hours and were subsequently washed five times in PBS to discard unbound material. The coverslips were then fixed by addition of formaldehyde (3.7% in PBS). Following fixation, the cells were incubated for 30 min. in PBS-BSA(0.1%) containing Texas Red-conjugated phalloidin to stain the actin cytoskeleton. The coverslips were then washed twice with PBS and were subsequently mounted in Vectashield containing DAPI (to stain cell nuclei) and analyzed using a Leica DM-IRBE fluorescence microscope. Quantification of aggregate binding to cells was performed as follows: Ten random fields were selected in a blind manner (i.e., without knowledge of the coverslips' identity) in the red channel (actin). Images were subsequently automatically taken in all three channels by using Qfluoro software. The images were then analyzed by assessing the number of FITC-labeled aggregates/field by using LEICA Qwin software. Alternatively, the samples were analyzed by confocal microscopy (LEICA) and images were processed using LEICA software.

Analysis of cell death

After exposure to endostatin or Aβ (25 µM, 24h) cells in the culture medium were collected and the remaining adherent cells were trypsinized and added to the detached cells in the medium thus obtaining the total pool of adherent and detached cells. Subsequently, the cells were stained with 0.02% Trypan blue and the percentage dead (Trypan blue-positive) cells was assessed using a Bűrker glass counter chamber. Triplicate samples were analyzed and 200 cells were counted in each sample.

Annexin-V labeling

Cells were grown on glass coverslips and, following exposure to either soluble or aggregated (non-fluorescent) endostatin or Aβ (25 µM, 24 h), were fixed in the culture

medium using 3.7% formaldehyde. Subsequently, the cells were analyzed for the presence of exposed phosphatidyl-serine on the cell surface using FITC-labeled annexin V (Sigma) according to the manufacturer's protocol.

Results and discussion

Bacterial endostatin forms amyloid fibers with cross-β structure

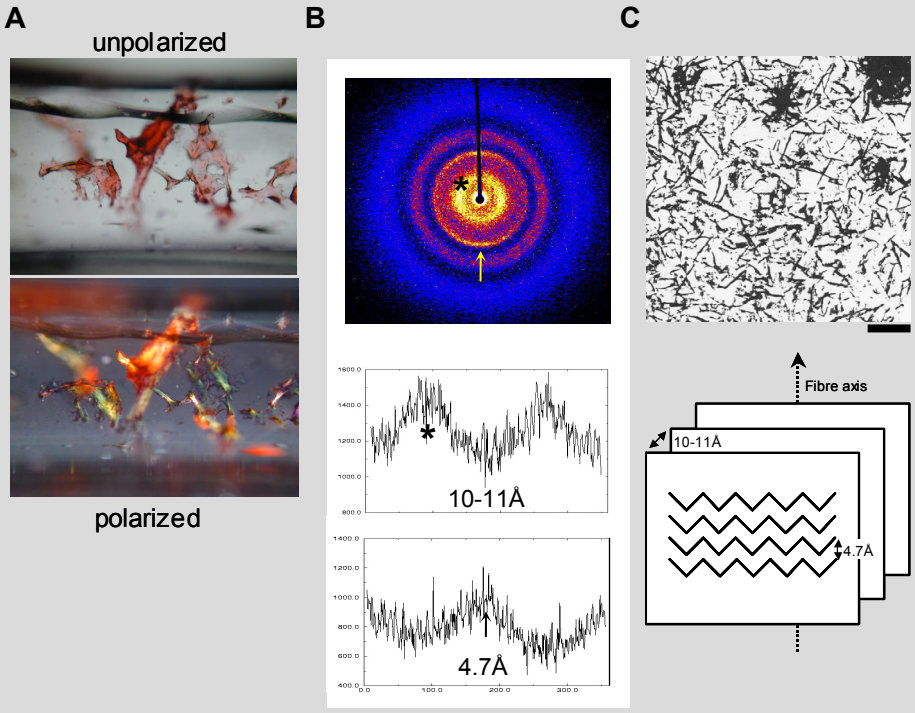
In solution, properly folded proteins or peptides form a stable three-dimensional structure. However, protein fragments (like endostatin) are often prone to (partial) denaturation. As a result, they may aggregate and become insoluble. Insoluble proteins may either exist as amorphous aggregates or as highly ordered amyloid deposits[18]. The latter structure results from extensive 'cross-β sheet' formation[19]. In cross-β sheets the polypeptide backbones are in a β-sheet conformation with hydrogen bonds between the separate polypeptide backbones. Congo Red is a dye that is used for the detection of cross-β sheet-forming amyloid deposits, showing green birefringence under polarized light[20]. We found that insoluble endostatin, as it is produced from bacteria, binds Congo Red (Figure 1a). Furthermore, when examined under polarized light it exhibits the green birefringence that is characteristic for Congo Red-bound amyloid deposits.

Next, we investigated whether cross-β sheets are indeed present in bacterial endostatin by performing X-ray diffraction analysis[21]. We found that the bacterial endostatin sample produced distinct reflection lines at 4.7 Å (hydrogen-bond distance), as well as at 10-11 Å (inter-sheet distance) (Figure 1b). It is important to note that the reflection lines at 4.7 Å and 10-11 Å show maximal intensities at opposite diffraction angles (Figure 1b). The fiber axis with its 4.7 Å hydrogen bond repeat distance is oriented along the vertical capillary axis. This implies that the inter-sheet distance of 10-11 Å is perpendicular to these hydrogen bonds in the protein aggregates. This is consistent with the protein being in a cross-β sheet conformation (See also Kranenburg et al.[22]). Intramolecular β-sheets in a globular protein cannot cause a diffraction pattern that is so ordered. A rough estimate of 54 Å for the crystallite size in the hydrogen bond direction is obtained from the width of the 4.7 Å reflection. This corresponds to about 11-12 β-strands connected by hydrogen bonds. The scattering of the 10-11 Å repeat is broad as usual. This is due both to the limited crystallite size in the inter-sheet direction and to variation in the inter-sheet distance. From the amount of background scattering it follows that only part of the protein is involved in cross-β sheet formation.

Proteins and peptides that form cross-β sheets have the tendency to aggregate into fibrillar structures that can be visualized by transmission electron microscopy (TEM)[23]. Therefore, we examined the endostatin aggregates using TEM. Figure 1c shows that bacterial endostatin forms unbranched fibers with a diameter of approximately 300 Å (30 nm) and with lengths varying from 1500-5000 Å (15-500 nm). Characteristic amyloid peptides form fibrils with diameters ranging from 50-130 Å (5-13 nm)[18] and with varying lengths up to 1 µm. Taken together, our results show that bacterial endostatin is a protein fragment with an

inherent propensity to form cross-β sheets and to aggregate into relatively thick amyloid-like fibrils.

Figure 1.

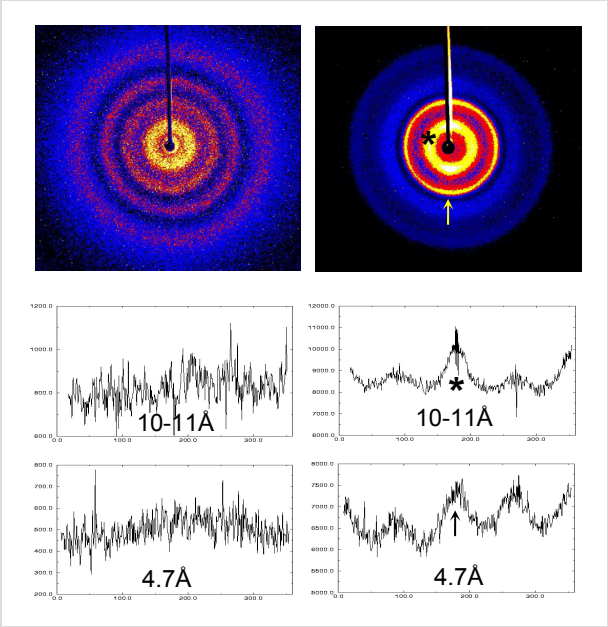


Amyloid properties of endostatin produced in bacteria. (A) Endostatin was purified from bacteria and stained with Congo Red. Samples were analyzed by light microscopy using both polarized and non-polarized light. The figure shows that endostatin binds Congo Red and exhibits green birefringence when examined under polarized light. (B) Endostatin samples were further analyzed by X-ray diffraction. The scattering as obtained on the 2D detector is color-coded by intensity on a linear scale. The scattering intensity decreases as white-yellow-red-blue-black. The pattern shows diffraction maxima at 4.7 Å and 10-11 Å. The fiber axis (hydrogen bond direction) with 4.7 Å repeat is oriented vertically and is indicated by the arrow. The 10-11 Å repeat is preferentially oriented perpendicular to that as indicated by the asterisk. Tangential scans along the 2θ scattering angles corresponding to both d-spacings in the lower panel show that the scattering at 4.7 Å is oriented vertically and that at 10-11 Å horizontally. (C) Endostatin samples were coated on a 400 mesh grid and were processed for transmission electron microscopy. Endostatin forms fibrils with a diameter of ~30 nm and lengths ranging from 150 to 500 nm. (D) Model of the endostatin fiber. The measured distances between individual peptide backbones (H-bonds) and between the distinct sheets are indicated, as well as the fiber axis (see also[17]). A rough estimate of 54 Å for the crystallite size along the fiber axis corresponds to about 11-12 β-strands connected by hydrogen bonds.

Conversion of soluble yeast-produced endostatin into amyloid endostatin

In contrast to bacterial endostatin, endostatin produced in yeast is soluble, and neither binds Congo Red, nor forms amyloid fibrils when examined using TEM (not shown). A major difference in the isolation protocols is a denaturation step using 8 M urea during the isolation of endostatin from bacteria, but not from yeast. Since amyloid formation occurs via (partially) denatured intermediates[18], we considered the possibility that this step may allow for the efficient stacking of endostatin monomers to form cross-β sheets. Therefore, we subjected soluble yeast endostatin to the same denaturation/renaturation protocol and examined the resulting preparation for cross-β sheet content using X-ray diffraction. Figure 2a shows that soluble endostatin produced in yeast does not show any signs of cross-β sheet formation. The diffraction pattern is typical for any amorphous globular protein and does not show a sharp reflection line at 4.7 Å. Furthermore, there is no perpendicular orientation of the diffuse reflection lines at 4.7 Å and 9-11 Å. The X-ray diffraction data are in line with our findings that soluble endostatin does not bind to Congo Red and does not form fibrils. However, after denaturation/renaturation we found extensive cross-β sheet formation in yeast-produced endostatin (Figure 2b). In addition to urea treatment, protein denaturation through freeze-thawing or heating also induced endostatin aggregation (not shown). We conclude that endostatin is a protein with a high propensity to form amyloid aggregates, a

Figure 2.



Conversion of soluble globular endostatin into amyloid endostatin. Endostatin produced in yeast was dialyzed against 8 M urea followed by extensive dialysis against H_2O. During dialysis endostatin precipitates as a fine white solid. The samples were processed for X-ray diffraction analysis. (A) Diffraction pattern of untreated (yeast-produced) endostatin with no sign of cross-β sheet structure. (B) Diffraction pattern of urea-treated endostatin with extensive cross-β sheet formation. During solvent evaporation fibril formation occurred both vertically and horizontally in the capillary, as evidenced by the occurrence of peaks at 90, 180, 270 and 0/360 degrees in the tangential scans corresponding to both d-spacings. The asterisk indicates the peak reflection of the 10-11 Å d-spacing at 90^0. The arrow indicates the peak reflection of the 4.7 Å d-spacing at 180^0.

process that is greatly enhanced when the protein undergoes (partial) denaturation. In this light it is interesting to note that the β-sheet content measured in the endostatin crystal (25%) does not match the percentage of β-sheet content of the original solution (70%)[17;24]. This implies that the solution from which the crystal has grown contained a β-sheet-rich form(s) of endostatin that did not crystallize. Due to the intrinsic heterogeneity of cross-β sheet forming proteins, crystallization of such structures is notoriously difficult.

Binding of endostatin to N1E-115 cells requires cross-β structure

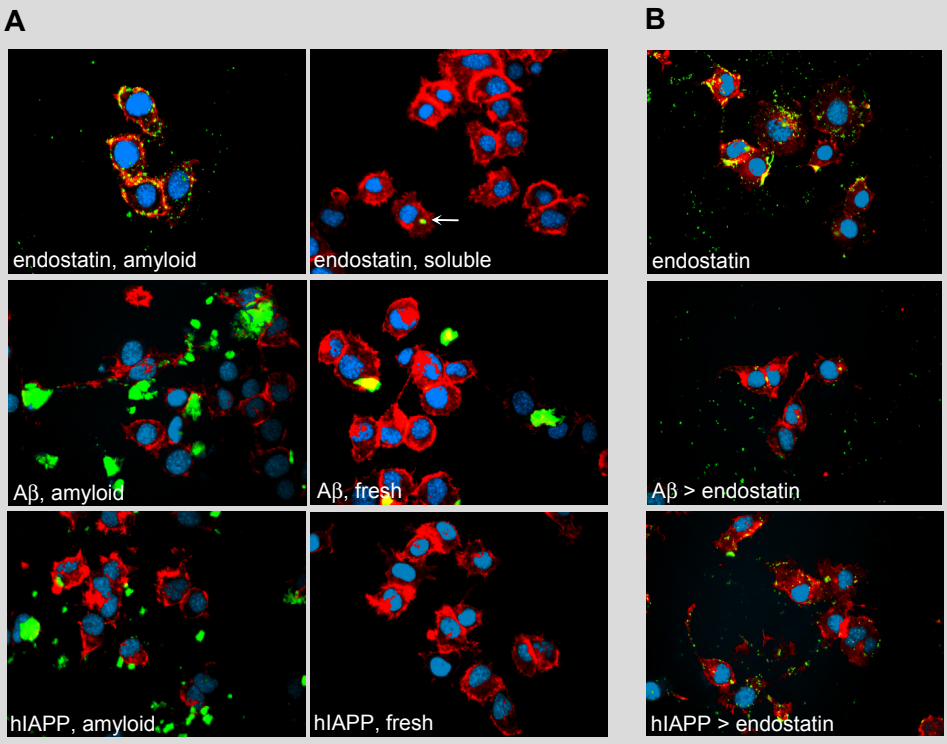
The prototype amyloid protein is Aβ, a 40-42 amino-acid peptide that is found as insoluble aggregates in neuronal tissue and in the brain microvasculature of Alzheimer's disease patients[25]. Like endostatin and many other amyloid proteins or peptides, Aβ is a naturally occurring cleavage product of a larger precursor protein[25]. *In vitro*, Aβ has toxic effects on both endothelial and neuronal cell types[26-31].

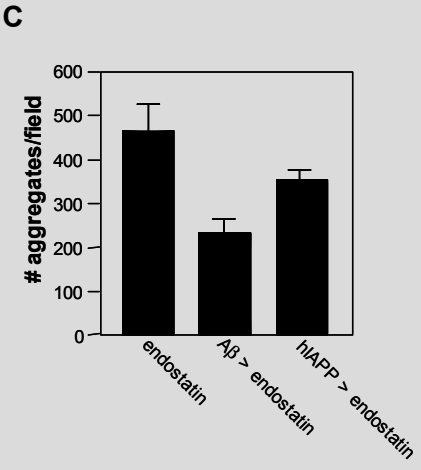
Based on the observed structural similarities between endostatin and Aβ we investigated whether endostatin would bind to and be toxic to neuronal cells. To this end we used the N1E-115 murine neuroblastoma cell line that can differentiate into post-mitotic neurons *in vitro*[32]. First, we tested whether endostatin can bind to these cells. Soluble endostatin was labeled with FITC and was either left untreated or was treated to form amyloid aggregates as above. As controls we also used FITC-labeled amyloid β (Aβ) and human islet amyloid polypeptide (hIAPP). The latter peptides were used in two structural conformations: freshly resuspended (non-cross-β) and pre-aggregated (cross-β). Cells were exposed to soluble or aggregated endostatin, to Aβ or to hIAPP for 1 hour. Following extensive washing and fixation, binding of endostatin, Aβ and hIAPP to the cells was assessed by fluorescence microscopy. Figure 3a shows that soluble endostatin is found neither associated to the cells, nor inside the cells, nor on the matrix surrounding the cells. In contrast, the majority of amyloid endostatin is cell-associated. Some of the deposits localized to the matrix, and some were found inside the cells (see below). Like amyloid endostatin, both Aβ and hIAPP readily bound to the cells. Two distinct types of aggregates were observed. Small endostatin-like aggregates as well as larger aggregates that are not observed in the endostatin preparation. In the freshly resuspended (non-cross-β) peptides the small aggregates were not observed but larger aggregates were occasionally found to be cell-associated (Figure 3). It is well known that the formation of extensive cross-β structure in these peptide aggregates may take days-weeks.

When using high concentrations of soluble endostatin, we observed occasional protein aggregation in the tissue culture medium and these aggregates were found to bind to the cells (Figure 3a upper panel, arrow). This indicates once again the propensity of soluble endostatin to undergo structural changes leading to amyloid aggregation. Factors present in the medium or in the serum may promote this conversion.

We reasoned that if cross-β structure underlies the binding of aggregated endostatin to the cells, amyloid β and hIAPP may compete for endostatin binding. We tested this by

Figure 3.

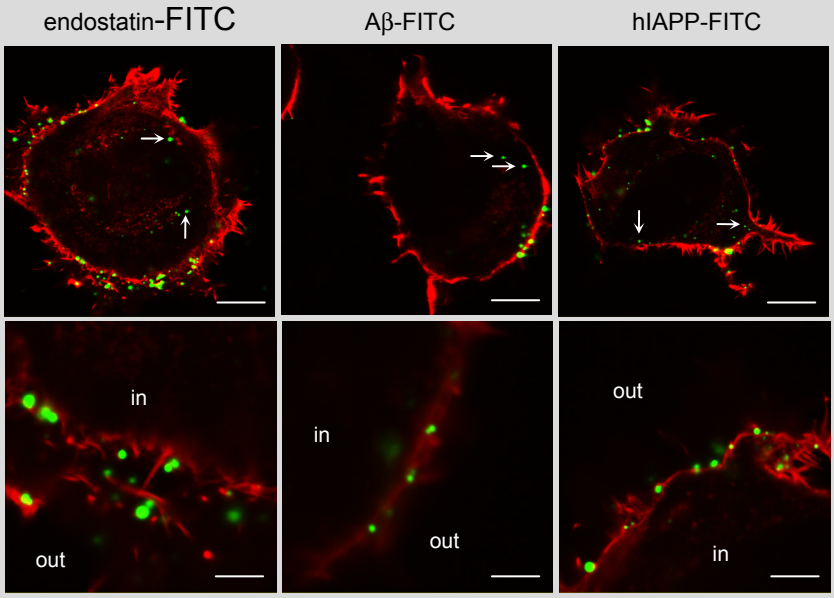




Binding of amyloid aggregates to N1E-115 neuroblastoma cells. (A) Neuronal N1E-115 cells, were incubated with either FITC-labeled aggregated amyloid endostatin, soluble endostatin, Aβ freshly resuspended, Aβ pre-aggregated for three weeks (Aβ amyloid), hIAPP freshly resuspended and hIAPP pre-aggregated for three weeks (hIAPP amyloid) (all at 5 μM) and washed. after 1 hour. The actin cytoskeleton was stained using Texas Red-conjugated phalloidin (red) and the DNA with DAPI (blue). All amyloid aggregates (left panels) bound N1E-115 cells. (B) N1E-115 cells were pre-incubated for 6 hours with non-fluorescent Aβ or hIAPP (at 5 μM) and the binding of FITC-labeled amyloid endostatin (1 hour, 5 μM) was subsequently tested as above. (C) Digital image analysis shows that pre-incubation of the cells with Aβ and, to a lesser extent, hIAPP diminishes endostatin binding (to 50 and 25% respectively), suggesting that the aggregates have common cellular binding sites. The bar diagram shows means of 10 randomly selected fields in the red channel to avoid bias.

incubating the cells for 6 hours with either Aβ or with hIAPP prior to incubation with amyloid endostatin for 1 hour. The binding of endostatin to the cells was then assessed by fluorescence microscopy and subsequent digital image analysis. Figure 3b and 3c show that Aβ and, to a lesser extent, hIAPP compete with amyloid endostatin for binding to the N1E-115 cells. Thus, amyloid endostatin shares cellular binding sites with other amyloid peptides, even though these peptides and endostatin do not share any overt primary sequence homology. We next extended these observations by analysing the binding of endostatin, Aβ and hIAPP to N1E-115 cells in more detail. To this end we allowed binding of the FITC-labeled aggregates to the cells as above and, following extensive washing and staining of the actin cytoskeleton with Texas Red phalloidin, the cell-bound aggregates were examined by confocal microscopy. Figure 4 shows images of single cells and of highly zoomed fragments of the cell surface in which discrete single aggregates are visible. We found that the majority of all three amyloid aggregates are in close proximity to the cortical actin-cytoskeleton that is connected to the plasma membrane (Figure 4). In addition, some of the aggregates are found inside the cells (arrows), indicating that at least some internalization of the aggregates can take place.

Figure 4.



Localization of cell-associated amyloid aggregates. N1E-115 cells were grown on glass coverslips and were incubated for 1 hour with FITC-labeled amyloid endostatin, Aβ and hIAPP. Actin was visualized using Texas Red-conjugated phalloidin. The coverslips were analyzed by confocal microscopy. Whole cell images (upper panel) show localization of all three amyloids mainly to the cell surface. In addition, some of the aggregates show intracellular localization (arrows) (Bar, 10 μm). Zoomed images of cell surface areas (lower panel) shows that all three types of amyloid aggregates are in close proximity to the plasma membrane-bound cortical actin cytoskeleton (Bar, 2 μm).

Amyloid but not soluble endostatin is cytotoxic to N1E-115 neuroblastoma cells

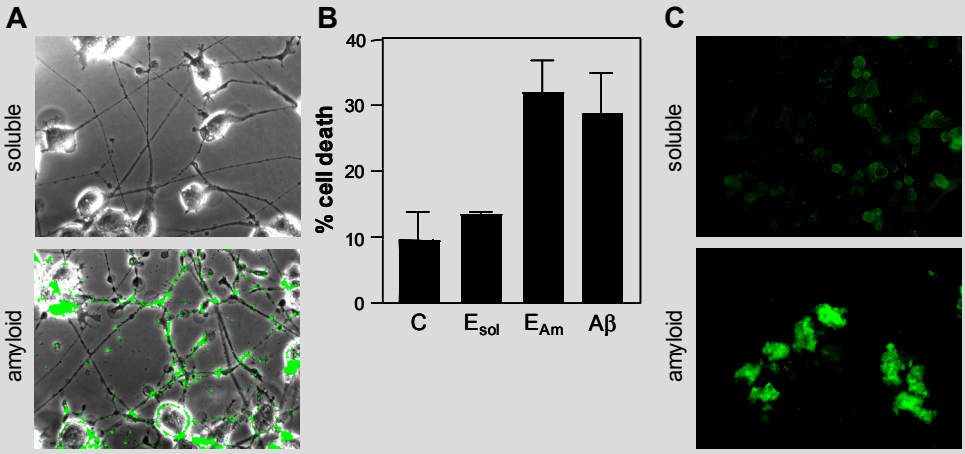
Next, we analyzed whether the distinct forms of endostatin would be cytotoxic to differentiated N1E-115 cells and compared their cytotoxicity with that induced by Aβ. Figure 5a shows that amyloid but not soluble endostatin binds to differentiated N1E-115 cells as it does to the undifferentiated cells (Figures 3,4). Furthermore, we found that a 24-hour exposure of these cells to either Aβ or to amyloid endostatin induced cell death, whereas soluble endostatin or buffer controls had no effect on neuronal cell viability (Figure 5). Amyloid peptides may induce either apoptosis or necrosis in neuronal and endothelial cells. Annexin V strongly binds to exposed phosphatidyl-serine, a marker for apoptotic cell death. We found that cells treated with amyloid endostatin, but not those treated with soluble endostatin, are highly positive for fluorescent annexin V (Figure 5c), indicating that endostatin-induced cell death is apoptotic in nature.

Interestingly, a recent report showed the production of endostatin by neuronal cells and the localization of endostatin to Aβ plaques in Alzheimer's disease brain[33]. However, the structural basis of this interaction was not examined. Given our finding that endostatin, like Aβ, has the propensity to form cross-β structure, a cross-β type interaction may account for their co-localization *in vivo*. Taken together, it seems likely that endostatin may affect neuronal cell function and survival, also *in vivo*.

In addition to using neuronal N1E-115 cells we also used bovine pulmonary aortic endothelial cells (BPAEC). We found that amyloid endostatin, but not soluble endostatin, is highly cytotoxic to these cells as it is to the neuronal cells. In contrast, neither soluble nor amyloid endostatin was cytotoxic to primary human umbilical vein endothelial cells (HUVEC) nor to human dermal microvascular endothelial cells (HDMEC)(not shown). At present we do not know what determines the sensitivity of (endothelial or neuronal) cells to amyloid endostatin. Possibly, amyloid endostatin may exert its toxic effects by activating amyloid receptors on the cell surface like receptor for advanced glycation end products (RAGE) or scavenger receptors like CD36[34;35].

Can amyloid toxicity explain the antiangiogenic effect of endostatin? We found the formation of liver metastases by C26 murine colon cancer cells to be sensitive to treatment with endostatin. In this model both soluble and amyloid endostatin inhibited tumor growth, but neither form caused tumor regression[36]. Given the propensity of soluble endostatin to aggregate and the inability to control this phenomenon *in vivo*, it is impossible to assign antitumor activity to a specific structural form of endostatin.

Figure 5.



Toxicity of amyloid endostatin. (A) N1E-115 cells were differentiated into post-mitotic neurons on glass coverslips and were subsequently treated overnight with either FITC-labeled soluble or with FITC-labeled amyloid endostatin. Following extensive washing and fixation, binding of endostatin to the cells was visualized with fluorescence microscopy. Merged bright field and fluorescence images are shown. Only amyloid endostatin binds to the differentiated neuronal cells. (B) Differentiated N1E-115 cells were treated for 24 hours with either buffer, soluble endostatin (25 μM) (E_{sol}), amyloid endostatin (25 μM) (E_{Am}), or with Aβ (25 μM). Cell viability was subsequently assessed by Trypan blue exclusion, counting both adherent and detached cells. The bar diagram shows means of triplicate samples. (C) N1E-115 cells were differentiated on glass coverslips and were subsequently treated with either soluble or with amyloid endostatin. Following fixation, the cells were stained with annexin V-FITC to visualize exposed phosphatidylserine. Detaching cell clusters that are associated with endostatin aggregates are highly positive for annexin V.

Concluding remarks

Although many reports have shown effects of either soluble or insoluble endostatin on cell behavior[3], it is far from clear which mechanisms underlie which phenomena. Induction of apoptosis and inhibition of cell migration seem to be the most commonly found cellular effects[3]. Our results provide an explanation for the observed cytotoxic effects of endostatin. Amyloid formation often occurs in protein fragments that are taken out of their natural context (i.e. the full length protein), presumably due to partial denaturation[18]. The hydrophobicity of the peptide sequence and the propensity of the sequence to form β-sheets are critical determinants of protein aggregation[37]. Recently it was found that two unrelated protein fragments (which, unlike Aβ are not related to any disease) become highly toxic upon aggregation[38]. Taken together, it seems likely that protein aggregation *per se*, independent of the primary amino-acid sequence, endows aggregated amyloid proteins with

an inherent toxicity[38]. The results presented here suggest that endostatin can be added to the list of 'toxic-when-aggregated' proteins. It is to be expected that this list will become much longer in the near future. It is important to note that the extent of toxicity is determined to a large extent by the level of aggregation and the structural basis of aggregation[38]. These phenomena, in turn, greatly depend on a number of parameters including protein production and storage protocols, pH, and choice of solvents.

Our results show that endostatin-induced cytotoxicity is restricted to the aggregated amyloid form. Endostatin is toxic to endothelial[7-9] and neuronal (this study) cells. These cell types are also particularly sensitive to amyloid deposits[26-31]. Our finding that endostatin is a protein with amyloid properties may therefore explain the cell-type specificity of its cytotoxicity. If endostatin exerts its effect through cellular receptors, its bioactivity will depend on the expression of such receptors on the target cells. Therefore, we are presently studying whether endostatin can activate receptors that are known to bind to cross-β sheet peptides.

Acknowledgements

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Chapter 7

Tissue-type plasminogen activator (tPA) is a multiligand cross-β sheet receptor

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Summary

Tissue-type plasminogen activator (tPA) regulates fibrin clot lysis by stimulating the conversion of plasminogen into the active protease plasmin[1]. Fibrin is required for efficient tPA-mediated plasmin generation and thereby stimulates its own proteolysis. Several regions in fibrin can bind to tPA[1], but the structural basis for this interaction is not known. Amyloid β (Aβ) is a peptide aggregate that is associated with neurotoxicity in Alzheimer's disease brain[2]. Like fibrin, it stimulates tPA-mediated plasmin formation[3-5]. Intermolecular stacking of peptide backbones in β-sheet conformation underlies cross-β structure in amyloid peptides[6]. We show here that fibrin-derived peptides adopt cross-β structure and form amyloid fibers. This correlates with tPA binding and stimulation of tPA-mediated plasminogen activation. Prototype amyloid peptides including Aβ and islet amyloid polypeptide (IAPP, associated with pancreatic β cell toxicity in type II diabetes[7]) have no sequence similarity to the fibrin peptides but also bind to tPA and can substitute for fibrin in plasminogen activation by tPA. Moreover, the induction of cross-β structure in an otherwise globular protein (endostatin) endows it with tPA-activating potential. Our results classify tPA as a multiligand receptor and show that the common denominator in tPA-binding ligands is the presence of cross-β structure.

Materials and methods

Peptides

The following peptides were synthesized (Pepscan Systems, Lelystad, The Netherlands):
Fibrin peptides: FP13: KRLEVDIDKIRS; FP10: KRLEVDIDIK;
Aβ: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV;
hIAPP: KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY;
chIAPP: SNNFGAILSS; cmIAPP: SNNLGPVLPP.
Purity of the peptides was analyzed both by mass spectroscopy and by using high performance liquid chromatography (HPLC). The freeze-dried peptides were resuspended in phosphate-buffered saline (PBS). The short peptides FP13 and chIAPP immediately form cross-β sheet structure following resuspension as judged by CD spectroscopy and Congo Red binding. Cross-β sheet formation in the longer peptides Aβ and hIAPP required incubation at room temperature for three weeks.

Endostatin

Soluble recombinant globular human endostatin was kindly provided by Dr. Kim Lee Sim from Entremed Inc. (Rockville, Maryland, USA). Insoluble recombinant human endostatin was prepared from bacteria exactly as described[8].

Plasmin activity assay

Plasminogen (1 μM) was incubated with tPA (200 pM) and the individual peptides (5 μM) or the control buffer without cofactor. Samples were taken from the reaction mixture at the indicated time points and plasmin generation was stopped by addition of ε-aminocaproic acid (εACA) (0.5 M) and EDTA (0.5 M). The chromogenic substrate S-2251 (Chromogenix AB, Mölndal, Sweden) was then added to the samples and plasmin activity was assessed by on-line measurement of S-2251 conversion at 405 nm.

tPA activity assay

tPA, at the indicated concentrations, was mixed with the individual peptides (5 μM) or the control buffer without cofactor and the chromogenic substrate S-2765 (Chromogenix AB, Mölndal, Sweden). tPA activity was then assessed by on-line measurement of S-2765 conversion at 405 nm.

Solid phase binding assay

Peptide solutions were coated onto plastic 96-well plates. The plates were subsequently washed with phosphate-buffered saline (PBS) and were blocked in PBS containing 0.1% BSA. tPA was subsequently allowed to bind at the indicated concentrations. Binding of tPA to the peptides was assessed by using anti-tPA (PoAb 385R, American Diagnostica, Greenwich, CT) followed by horseradish peroxidase (HRP)-conjugated Swine-anti-Rabbit IgG (DAKO). Background tPA binding to the plastic wells was assessed in all experiments by omitting the tester peptides from the initial coating step. After extensive washing, HRP activity was measured by substrate (1,2-phenylenediamine) conversion and measurement of the OD at 490 nm. Background values were subtracted from the test values and K_d's were calculated from the binding curves by using GraphPad Prism software (GraphPad Software, San Diego CA, USA).

Congo red staining

Single drops of peptide solutions in H_2O were spotted onto glass coverslips and were subsequently air-dried. The peptide material was then stained with Congo Red (Sigma) according to the manufacturer's protocol.

Thioflavin T binding

Thioflavin T was added to the peptide solutions (0.1 mg/ml) in 50 mM glycine (pH 9.0) at the indicated concentrations. Following excitation at 435 nm fluorescence emission was recorded at 485 nm in a Hitachi F4500 fluorescence spectrophotometer. Averaged values are shown of three independent measurements.

Circular Dichroism measurements

Peptide solutions (0.1 mg/ml in H_2O) were analyzed in a JASCO 600 CD spectropolarimeter. Averaged absorption spectra are shown of 5 single measurements from 190 to 240 nm. Ellipticity ($Dg.cm^2/dmol$) is plotted against wavelength (nm).

Transmission electron microscopy

Peptide samples were applied to 400 mesh specimen grids covered with carbon-coated collodion films. After 5 min. the drops were removed with filter paper and the preparations were stained with 1% methylcellulose and 1% uranyl acetate. After washing in H_2O, the samples were dehydrated in a graded series of EtOH and hexanethyldisilazane. Transmission electron micrographs were recorded at 60 kV using a JEOL-1 electron microscope.

X-ray diffraction analysis

Peptide solutions (1 mg/ml) were taken up in a glass capillary. The water was then allowed to evaporate over a period of several days. The samples were placed in the capillary, on a Nonius κCCD diffractometer. Scattering was measured using sealed tube MoKα radiation with a graphite monochromator on the CCD area detector for a period of 16 hours. Scattering from air and the capillary glass wall were subtracted using in-house software (VIEW/EVAL). The crystallite size was determined by the Scherrer formula $D_{av}=(0.89\lambda)/(\beta\cos\theta_{max})$, in which D_{av}=average crystallite size in Á; λ=wavelength; β=2θ width at half maximum value in radians.

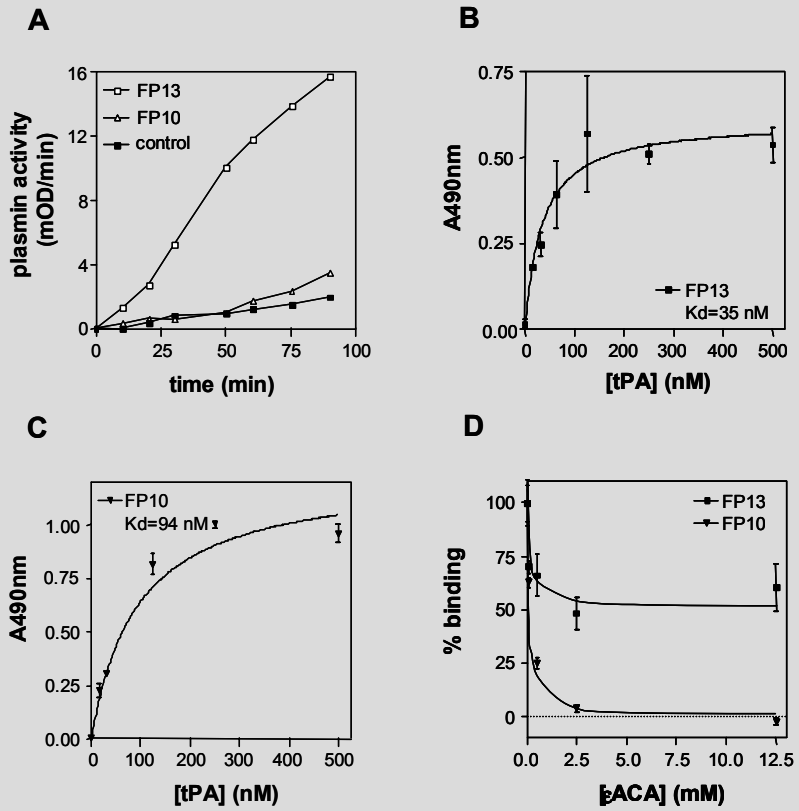
Results

tPA-mediated plasminogen activation by fibrin-derived peptides

One of the amino acid sequences within the fibrin β-chain that supports tPA binding and activation encompasses the 13 residues 148-160[1]. Based on the amino acid sequence of this region, we made two peptides: Fibrin-derived peptide 13 (FP13) (148-160: KRLEVDIDIKIRS) and FP10 (148-157: KRLEVDIDIK). First, we tested the capacity of the peptides to substitute for fibrin in stimulating tPA-mediated plasminogen activation. Addition of FP13, but not FP10, to a mixture of plasminogen and tPA lead to a potent increase in the generation of plasmin activity in time (Figure 1a).

Next, we studied the ability of the peptides to bind to tPA by performing solid-phase binding assays. Surprisingly, we found that both peptides bound to tPA despite the fact that only FP13 activates tPA (Figure1 a-c). Since FP10 has a carboxy-terminal lysine residue (K), binding to this peptide could be mediated by the kringle domain(s) in tPA. In line with this notion, binding of FP10 to tPA is completely lost in the presence of the lysine analogue ε-aminocaproic acid (εACA) (Figure 1d). In contrast, εACA inhibits the binding of tPA to FP13 by only about 40%, indicating that this interaction is largely lysine-independent (Figure 1d).

Figure 1.



tPA binding and plasminogen activation by fibrin-derived peptides. (A) Plasminogen (0.1 mg/ml) and tPA (200 pM) were incubated with the fibrin-derived peptides (5 μM) or with control buffer. The conversion of plasminogen into plasmin was followed over a period of time by measuring plasmin activity using a chromogenic substrate (S-2251). (B) Binding of FP13 to tPA. FP13 was coated onto plastic and was overlayed with the indicated concentrations of tPA. tPA binding was then assessed by using the polyclonal anti-tPA antibody 385R in an ELISA-type assay. (C) Binding of FP10 to tPA was assessed as in (B). (D) tPA binding to FP13 and FP10 was assessed as in (B) and (C) using 200 nM tPA in the presence of the indicated concentrations of εACA.

Fibrin-derived peptides form cross- β sheets

The above experiments show that the binding of tPA to the two peptides occurs through distinct types of interaction, one supporting tPA-mediated plasminogen activation, the other not. We considered the possibility that differences in the peptide structure could underlie the

difference in cofactor activity. The relative contribution of random coils, β-sheets and α-helices to the structure of the peptides in solution was studied by circular dichroism measurements. Strikingly, we found that FP13 was in a 100% β-sheet conformation with a characteristic minimum at 215 nm and a maximum at 190 nm (Figure 2a). In contrast, FP10 was completely random coiled with a minimum observed at 198 nm (Figure 2a). Therefore, it seems likely that structural differences underlie the differences in ability of these peptides to activate tPA.

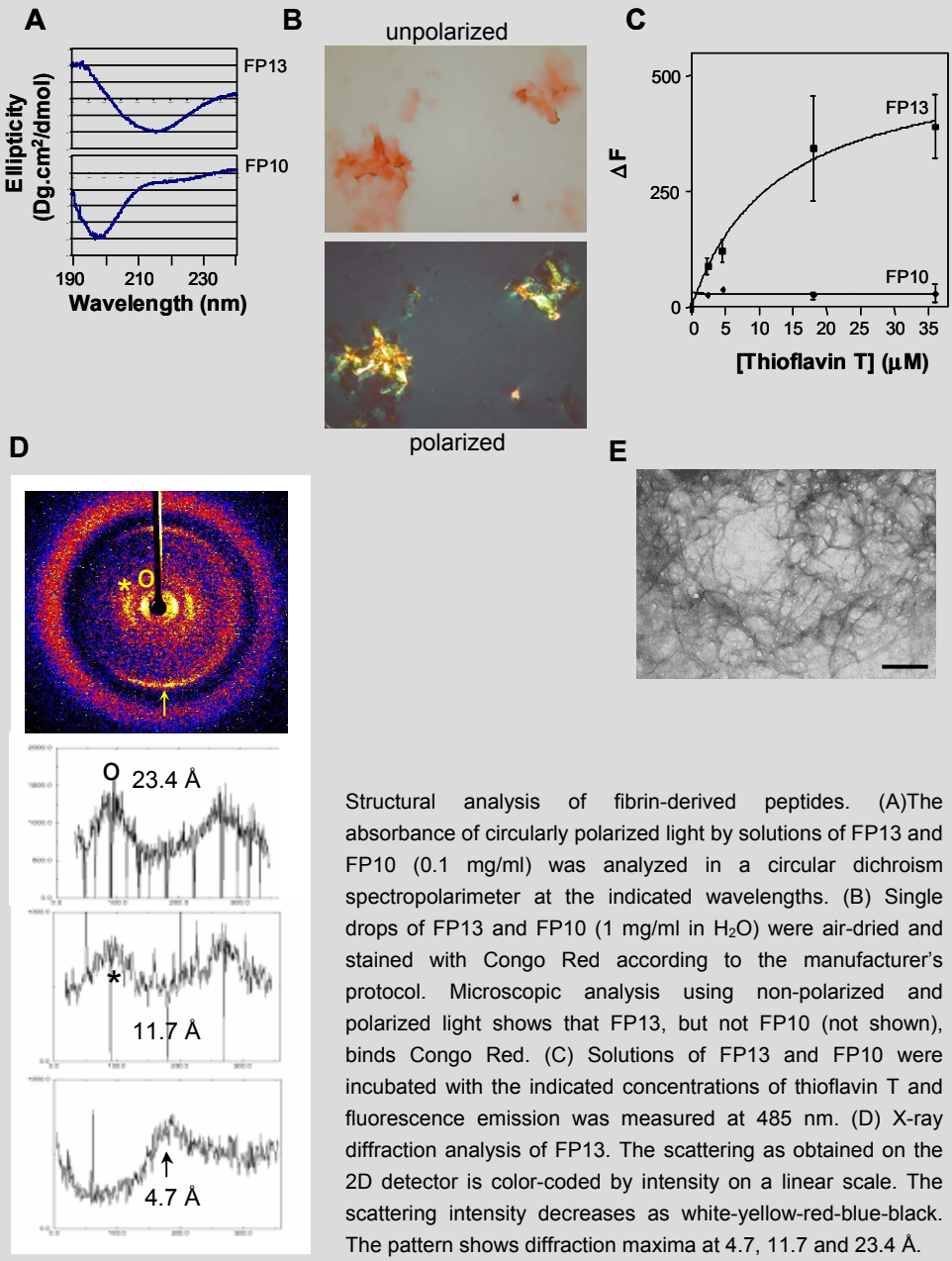
Given the high β-sheet content in FP13 and given the finding that amyloid β (Aβ) supports plasminogen activation by tPA[3-5], we tested whether FP13 is an amyloid peptide with cross-β sheet conformation. Congo Red binds to amyloid peptide aggregates, irrespective of the amino acid sequence[9]. Dried samples of FP10 and FP13 were stained with Congo Red and examined by light microscopy. Figure 2b shows that FP13 dried as aggregates that readily bound Congo Red, showing the characteristic green birefringence when examined under polarized light. In contrast, FP10 dried as a uniform film that did not bind Congo Red (not shown).

Thioflavin T (ThT) is a fluorescent indicator of cross-β sheets in solution[9]. By incubating the peptide solutions with increasing concentrations of ThT and subsequent measurement of fluorescence emission, we found that FP13, but not FP10, enhanced ThT fluorescence (Figure 2c). Thus, the high content of β sheets in FP13 presumably reflects cross-β sheet conformation. The presence of cross-β sheets can be demonstrated by X-ray diffraction analysis[10]. When analyzed by X-ray diffraction (Figure 2d), FP13 caused a distinct diffraction pattern, with reflection maxima at 4.7, 11.7 and 23.4 Å. The fiber axis with its 4.7 Å hydrogen bond repeat distance is oriented along the vertical capillary axis. The 11.7 Å repeat is oriented perpendicular to that. The perpendicular orientation of the 4.7 Å and 11.7 Å distances is typical for cross-β sheets. The 23.4 Å reflection that, like the 11.7 Å reflection, is oriented horizontally, signals higher ordering in the inter-sheet direction. Rough estimation of the crystallite sizes from the width of the reflections gives 120 Å for the fiber direction and 40-50 Å for the inter-sheet direction. This amounts to approximately 25 β-strands being hydrogen-bonded into an array of about 4 cross-β sheets.

As a result of cross-β sheet formation, peptides may undergo fibrillogenesis[6] and such fibers can be visualized using transmission electron microscopy (TEM). Single drops of peptide solutions were therefore examined by TEM. We found that FP13, but not FP10 (not shown), formed unbranched fibrils that were about 5 nm thick with lengths ranging from 250-1000 nm (Figure 2e). These dimensions are within the range of dimensions found for other amyloid peptides[11].

Thus, FP13 is an amyloid peptide with cross-β sheet conformation, it binds to tPA and it stimulates tPA-mediated plasminogen activation. In contrast, FP10 is in a random coil conformation and binds to tPA through its carboxy-terminal lysine residue without stimulating tPA-mediated plasmin formation.

Figure 2.



Structural analysis of fibrin-derived peptides. (A)The absorbance of circularly polarized light by solutions of FP13 and FP10 (0.1 mg/ml) was analyzed in a circular dichroism spectropolarimeter at the indicated wavelengths. (B) Single drops of FP13 and FP10 (1 mg/ml in H_2O) were air-dried and stained with Congo Red according to the manufacturer's protocol. Microscopic analysis using non-polarized and polarized light shows that FP13, but not FP10 (not shown), binds Congo Red. (C) Solutions of FP13 and FP10 were incubated with the indicated concentrations of thioflavin T and fluorescence emission was measured at 485 nm. (D) X-ray diffraction analysis of FP13. The scattering as obtained on the 2D detector is color-coded by intensity on a linear scale. The scattering intensity decreases as white-yellow-red-blue-black. The pattern shows diffraction maxima at 4.7, 11.7 and 23.4 Å. In the lower panels, tangential scans along the 2θ scattering angles corresponding to all three d-spacings show that the 4.7 Å scattering (hydrogen bonds) is oriented perpendicular to those at 11.7 and 23.4 Å (1x and 2x inter-sheet distance). (E) Solutions of FP13 and FP10 were spotted on a carbon-coated grid and analyzed by transmission EM. FP13, but not FP10, shows unbranched amyloid-like fibers. Bar = 200 nm.

tPA binding and plasminogen activation by cross-β sheet peptides irrespective of their amino acid sequence

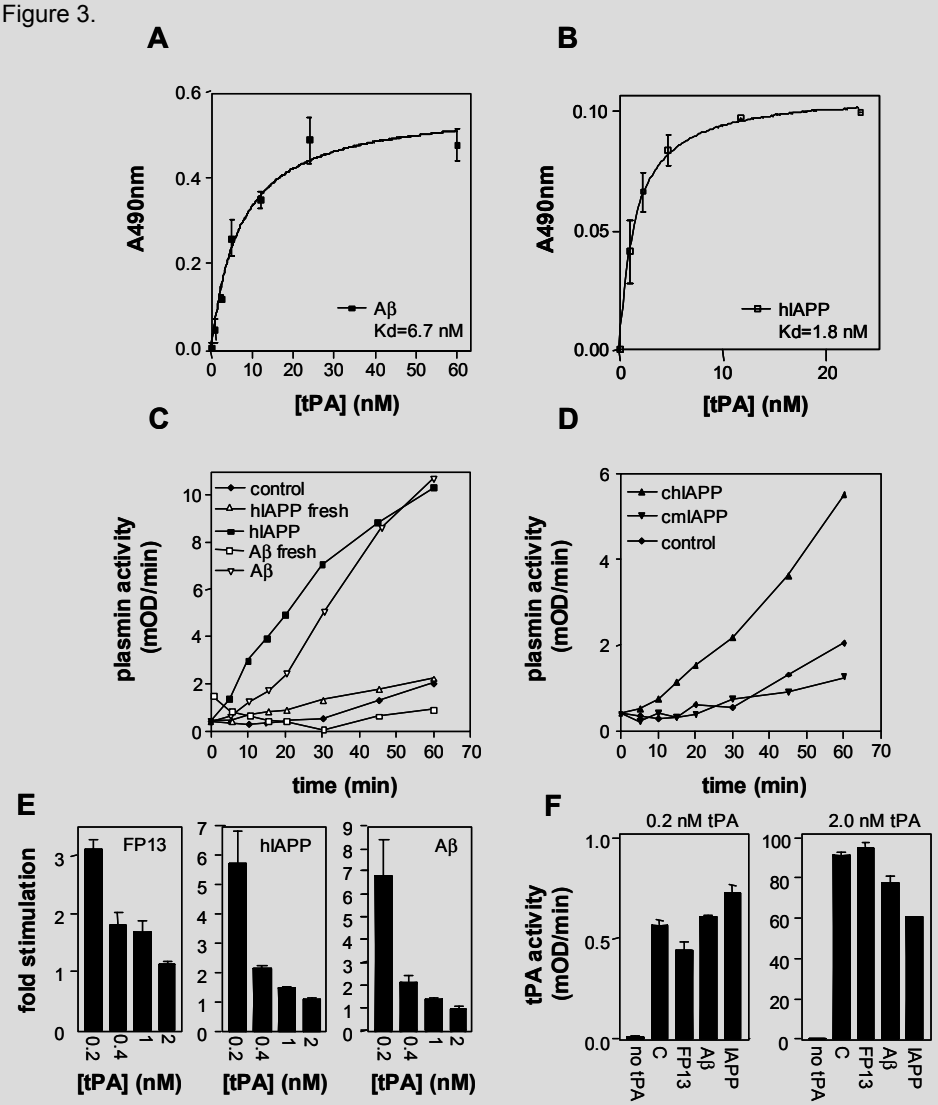
The above results prompted us to investigate the binding characteristics of several amyloid peptides to tPA. Aβ stimulates tPA-mediated plasmin formation[3-5] but, to our knowledge, no binding data have been published. To test tPA binding to Aβ and to other amyloid peptides, we performed solid-phase binding assays and detected bound tPA by ELISA. Figure 3a shows that tPA binds to Aβ with high affinity (6.7 nM) as it does to the fibrin peptides. Human Islet Amyloid Polypeptide (hIAPP) is a 37-amino acid peptide with amyloidogenic properties that is found deposited in the Islets of Langerhans in the pancreas of type II diabetic patients. It acts as a diabetogenic factor, presumably by being toxic to the insulin-producing β-cells[7]. The hIAPP amino acid sequence is unrelated to that of Aβ or the fibrin peptides. We found that, like Aβ and the fibrin peptides, hIAPP binds to tPA with high affinity (K_d=1.8 nM) (Figure 3b).

Next, we tested the capacity of these two distinct pre-aggregated amyloid peptides (Aβ and hIAPP) to substitute for fibrin in stimulating tPA-mediated plasminogen activation. We found that both peptides greatly enhance tPA-mediated plasmin formation (Figure 3c). Interestingly, the same peptides did not stimulate tPA-mediated plasminogen activation when used immediately after solubilization (Figure 3c). Thioflavin T binding to both peptides increased gradually over a period of three weeks (not shown). Thus, the formation of cross-β structure in the peptides is accompanied by an acquired ability to enhance tPA-mediated plasmin generation.

Whereas the rate of Aβ-stimulated plasmin formation is further enhanced during the reaction, that induced by hIAPP is not. We have found that during Aβ-stimulated plasmin formation limited proteolysis generates free internal lysines in Aβ (Kranenburg *et al.*, submitted). This may enhance the reaction by allowing more plasminogen and tPA binding. The lack of further rate-enhancement during reactions with hIAPP may be explained by the fact that it has only one lysine residue that is located at the extreme N-terminus.

One of the amyloidogenic regions in hIAPP encompasses amino acid residues 20–29[7;12-15]. Diabetic mice, in contrast to humans, do not develop pancreatic amyloid because mouse IAPP (mIAPP) has a different amino acid sequence in this region and lacks the propensity to adopt cross-β structure. We tested the presence of cross-β structure in these 'core' regions in human and mouse IAPP (chIAPP, cmIAPP) by Congo Red binding and compared their ability to stimulate tPA-mediated plasminogen activation. As expected, Congo Red readily bound chIAPP, but not cmIAPP, showing green birefringence under polarized light (not shown). Figure 3d shows that chIAPP stimulated tPA-mediated plasmin formation, albeit less efficiently than the full length IAPP, whereas cmIAPP was ineffective. Taken together with the data on the fibrin peptides, we have shown that four amyloid peptides (FP13, Aβ, hIAPP, chIAPP) stimulate tPA-mediated plasmin formation, whereas two non-amyloid peptides (FP10, cmIAPP) do not. Therefore, the results strongly suggest that tPA binding and cofactor activity requires the presence of cross-β structure in the peptides.

Figure 3.



tPA binding and stimulation of plasminogen activation by Aβ and hIAPP. The binding of tPA to (A) Aβ (B) hIAPP was tested as in Figure 1B. (C) The stimulation of tPA-mediated plasminogen activation by Aβ and hIAPP, either freshly resuspended or pre-aggregated for three weeks, was tested as in Figure 1A. (D) The activation of tPA by mouse and human "core" IAPP (cmIAPP, chIAPP) was tested as in Figure 1A. (E) FP13, hIAPP and Aβ were incubated with plasminogen and with increasing concentrations of tPA. Plasmin formation was then assessed as in Figure 1. The measured rates of plasmin generation in time with and without the peptides were then used to calculate the extent to which plasmin generation was stimulated by the peptides. These values were subsequently plotted against the tPA concentrations. The peptides loose their rate-enhancing effect at higher tPA concentrations. (F) tPA, either at 0.2 nM or at 2 nM, was incubated with FP13, Aβ and hIAPP together with S-2765, a chromogenic peptide substrate for tPA. tPA caused substrate conversion, but none of the cross-β peptides stimulated tPA enzymatic activity.

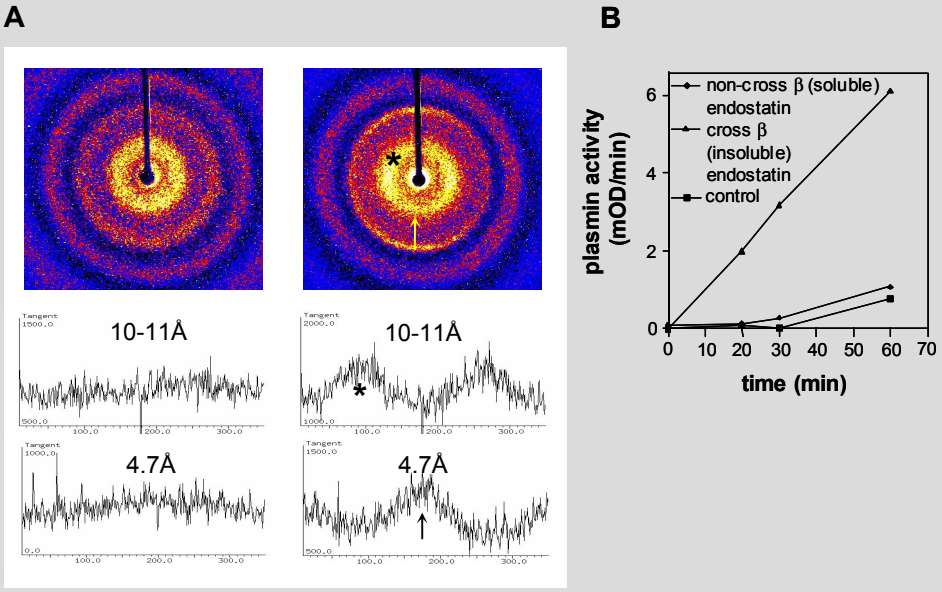
How do cross-β peptides cause enhanced plasminogen activation? Like fibrin, they could act as scaffolds by promoting the interaction between enzyme (tPA) and substrate (plasminogen). Alternatively, cross-β peptides may stimulate tPA enzymatic activity. With increasing tPA and plasminogen concentrations one would expect scaffold effects to diminish. In our assay, the tPA concentration is limiting. Therefore, we performed plasminogen activation assays with increasing concentrations of tPA, using FP13, hIAPP and Aβ as cofactors. Figure 3e shows that the stimulatory effect of the cross-β peptides on plasmin generation is lost with increasing concentrations of tPA. Next, we tested whether the cross-β peptides would stimulate tPA enzymatic activity. Figure 3f shows that none of the peptides tested (FP13, IAPP, Aβ) had a stimulatory effect on the conversion of the chromogenic peptide substrate S-2765 by tPA, neither at low (0.2 nM) nor at high (2 nM) concentrations of the enzyme. Thus, cross-β peptides act as scaffolds for efficient plasminogen conversion by tPA without modulating tPA activity.

Finally, we tested whether cross-β sheet formation in larger proteins would also support tPA-mediated plasminogen activation. Firstly, it should be noted that cross-β sheet formation occurs during the polymerization of fibrinogen into fibrin as measured by Raman spectroscopy[16] and that this is accompanied by tPA-activating potential. Secondly, we have recently found that endostatin, a 20 kDa fragment of the extracellular matrix protein collagen XVIII, has the propensity to form cross-β structure and to aggregate into amyloid deposits (Figure 4a). Endostatin can also be isolated in a soluble globular form that does not form cross-β sheets (Figure 4a). These two forms of endostatin allowed us to compare the capacity of a single larger protein (20 kDa) in two distinct conformations (cross-β versus non-cross-β) in the stimulation of tPA-mediated plasminogen activation. Figure 4b shows that endostatin with cross-β structure potently stimulates tPA-mediated plasminogen activation like all the other cross-β peptides, but that globular endostatin has no effect. Thus, the correlation between cross-β structure and the ability to stimulate tPA-mediated plasminogen activation holds for (short) peptides as well as for larger proteins.

Discussion

Our results provide evidence that tPA is a multiligand receptor for proteins that display cross-β structure. Upon binding to tPA, all cross-β ligands tested stimulate tPA-mediated plasmin formation. The tPA-binding peptide sequences in fibrin have been identified[1] but no structural data are available that show the basis of this interaction. Early data on the structural changes that are associated with the conversion of fibrinogen into fibrin show that this is accompanied by a general increase in β-sheet content and by the formation of hydrogen bonds between lateral fibrin molecules[16]. This type of interaction within the fibrin meshwork is similar to that underlying the cross-β sheet structure in amyloid peptide aggregates[6]. Furthermore, we show here that one of the isolated tPA-binding regions in fibrin that supports plasminogen activation has cross-β structure. Thus, also in fibrin, tPA may bind to cross-β sheet-forming regions. It has long been known that aged fibrin deposits

Figure 4.



Enhanced plasminogen activation by cross-β–structured endostatin. (A) X-ray diffraction analysis of two distinct forms of endostatin. The left panel shows globular endostatin with no detectable cross-β sheets. The right panel shows insoluble endostatin with abundant cross-β sheets. The scattering as obtained on the 2D detector is color-coded as in Figure 2D. The pattern shows diffraction maxima at 4.7 and 10-11 Å. The fiber axis (hydrogen bond direction) with 4.7 Å repeat is oriented vertically. The 10-11 Å repeat is preferentially oriented perpendicular to that, as indicated by the asterisk. Tangential scans along the 2θ scattering angles corresponding to both d-spacings in the lower panel show that the scattering at 4.7 Å is oriented vertically with the maximum intensity at 180^0 indicated by the arrow. The reflection at 10-11 Å is oriented horizontally with the maximum at 90^0 indicated by the asterisk. (B) Stimulation of tPA-mediated plasminogen activation by the two distinct forms of endostatin was assessed as in Figure 1A.

can bind Congo Red in tissue sections[17]. We have been able to corroborate these data *in vitro* by showing the selective staining of specific regions within a fibrin clot with Congo Red and by enhanced thioflavin T fluorescence during clot formation (O.K. and B.B., unpublished observations). In addition, serum amyloid P component (SAP), as well as Aβ itself, modulates the assembly and lysis of fibrin clots[18-20]. Taken together, the available data suggest that the control of fibrin assembly and fibrinolysis is regulated by cross-β structures and amyloid-binding proteins. The identification of tPA as a general cross-β sheet-binding molecule strongly reinforces this notion. In contrast to tPA, urokinase-type plasminogen

activator (uPA) is not activated by Aβ[4]. We are presently further investigating whether uPA is regulated by cross-β structure.

Like fibrin, partially denatured proteins can stimulate tPA-mediated plasminogen activation[21;22] and are prone to form cross-β sheets[11]. Our finding that the interaction of tPA with cross-β sheet peptides and proteins invariably leads to tPA-mediated plasminogen activation strongly suggests that these two phenomena are causally related. Thus, the formation of cross-β sheets endows protein aggregates (for instance endostatin) with tPA binding and plasminogen-activating potential. tPA may play a general role in the removal of cross-β sheet-containing (improperly folded) proteins by inducing their plasmin-mediated proteolysis. In this way tPA could prevent the accumulation of toxic protein aggregates in the circulation. Indeed, Aβ can induce its own destruction by activating the tPA/plasmin system in cultures of neuronal cells[23;24].

An important new insight is that protein aggregates, not necessarily related to any disease, are toxic to cells irrespective of their identity[25]. This implies that a common structural element rather than a specific amino acid sequence induces toxicity. Cross-β sheets are likely to represent such a sequence-independent structural element that can mediate toxic effects on cells. Hence, there must be molecular mediators of toxicity that recognize cross-β structure. With the discovery of tPA as a general cross-β sheet-binding protein we have identified a molecule that may contribute to the general cell toxicity induced by protein aggregates. Interestingly, tPA has been identified as an essential mediator of neuronal cell death following ischemia or excitotoxic injury in the brain[26;27]. By analogy, tPA may play a role in neuronal and endothelial cell death induced by Aβ and/or in IAPP-induced β-cell death.

Further work is needed to assess whether activation of the tPA/plasminogen system by protein aggregates (including IAPP and Aβ) prevents toxicity of the aggregates through their destruction, or whether tPA plays a role in mediating toxicity. These possibilities are not mutually exclusive. A critical variable between distinct aggregates will be their differential sensitivity to plasmin-mediated destruction.

Acknowledgements

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Chapter 8

Brief report: Fibrin Degradation Products induce endothelial cell detachment and inhibit tumor growth

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Summary

Inhibition of angiogenesis is an attractive approach to inhibit tumor growth. Several endogenously produced proteolytic fragments have been identified that inhibit angiogenesis. We recently demonstrated that the angiogenesis inhibitor endostatin is a cofactor for tPA-mediated plasminogen activation and induces tPA-mediated plasminogen-dependent cell detachment. During fibrinolysis tPA is activated by fibrin and fibrin fragments. Moreover, fibrin fragments are often found in the circulation of cancer patients. Therefore, we tested the hypothesis that fibrin fragments also induce endothelial cell detachment and inhibit tumor growth. We find that plasmin-generated fibrin degradation products (FDPs) induce detachment of bovine pulmonary endothelial cells and that FDPs inhibit the growth of subcutaneous tumors in mice. Our results support the idea that tPA-activating proteolytic fragments in general inhibit angiogenesis and tumor growth.

Introduction

The hemostatic system maintains the liquid flow of blood vessels by regulating fibrin deposition and platelet adherence. Upon vessel injury the coagulation system is activated and initiates the formation of polymerized fibrin through cleavage of fibrinogen by thrombin. Fibrin together with activated platelets forms a provisional barrier that occludes the vessel. Activation of the fibrinolytic system is needed for clot lysis and repair of injury. The serine protease plasmin is involved in fibrin degradation. Plasmin is formed upon cleavage of the zymogen plasminogen by plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). In contrast to uPA, tPA activity is stimulated by a cofactor. Fibrin and degraded fibrin are well known stimulators of tPA-mediated plasminogen activation.

The involvement of the hemostatic system in angiogenesis and cancer is becoming increasingly clear[1]. Fibrin as well as other extracellular matrix proteins that are part of a provisional matrix support endothelial cell attachment and facilitate migration and proliferation of endothelial cells. Platelets contain and secrete numerous regulators of angiogenesis[2]. Within the past decade proteins of the hemostatic system, and more particularly, cryptic fragments thereof, have been shown to inhibit tumor growth[1]. Examples of proteolytic fragments that inhibit tumor growth include angiostatin, endostatin, prothrombin fragments and anti-angiogenic antithrombin (chapter 9, table). Recently, we have shown that endostatin, a carboxy-terminal fragment of collagen XVIII, potently stimulates plasminogen activation by tPA and induces tPA-dependent plasminogen-mediated endothelial cell detachment[3;4]. Fibrin degradation products (FDPs) also stimulate plasminogen activation through tPA. Moreover, FDPs are present endogenously in the circulation of patients with cancer[5]. Here, we tested the hypothesis that fibrin fragments with tPA stimulatory activity are anti-tumorigenic[6].

Materials and methods

Reagents

Human fibrinogen and Thioflavin T were purchased from Sigma Chemical Co (St. Louis, MO). Human thrombin was from American Diagnostica (Greenwich, CT). tPA and plasmin substrate S-2251 were from Chromogenix AB (Mölndal, Sweden). Urokinase was from Calbiochem (La Jolla, CA).

Purification of proteins

Plasminogen was purified by a modification of the procedure of Deutsch and Mertz[33]. Human plasma was diluted three times in H_2O and passed through a lysine-Sepharose column equilibrated with 30 mM phosphate buffer (pH 7.4), 3 mM ethylenediaminetetraacetate (EDTA). After washing the column with 30 mM phosphate (pH 7.4), 500 mM NaCl, 3 mM EDTA, the plasminogen was eluted with 30 mM phosphate (pH

7.4), 200 mM ε-aminocaproic acid (εACA), 3 mM EDTA. εACA acid was removed by extensive dialysis against H_2O at 4°C and the plasminogen was stored at –80°C. Plasmin was produced as described in Walker and Nesheim[34]. In short, 1 mg/ml plasminogen in 50 mM Tris (pH 8.0), 150 mM NaCl, 25% glycerol, 50 mM εACA was activated by the addition of 50 U/ml urokinase at 37°C. The solution was loaded onto benzamidine-Sepharose and plasmin was eluted with 50 mM Tris (pH 8.0), 150 mM NaCl containing 20 mM benzamidine. Plasmin containing fractions were precipitated by dialysis against 80% saturated ammonium sulphate, resuspended in 50% glycerol and stored at –20°C. Plasmin was characterized using S-2251 and SDS-PAGE analysis.

Preparation of fibrin degradation products

Cross-linked fibrin was made by incubating 5 mg/ml fibrinogen with 8 nM thrombin at 37°C in the presence of 5 mM $CaCl_2$ for 5 hr. Fibrin degradation products were generated by digesting the fibrin clot with 35 nM plasmin overnight at 37°C. Plasmin was inactivated by addition of 1 mM diisopropyl fluorophosphate.

Endothelial cell culture

Primary endothelial cells, bovine pulmonary arterial endothelial cells (BPAEC), were obtained from the American Type Culture Collection (Rockville, MD). BPAEC were cultured in Dulbecco's Modified Essential Medium (DMEM) (Gibco Laboratories, Grand Island, NY) supplemented with 20% non heat-inactivated fetal calf serum (FCS) and antibiotics.

Plasminogen activation assay

Cofactor activity of FDPs was determined using a plasminogen activation assay. Different concentrations of FDPs were added to HBS buffer (20 mM Hepes, 4 mM KCl, 137 mM NaCl, 3 mM $CaCl_2$, 0.1 % BSA, pH 7.4) containing 50 µg/ml plasminogen. The reactions were started at 37°C by the addition of tPA at a final concentration of 30 U/ml. At different time points, 20 µl samples were taken and the reaction was stopped with 20 µl buffer containing 150 mM εACA, 150 mM EDTA. Plasmin activity was determined in 96-well plates by adding 20 µl chromogenic substrate S-2251 (2.5 mM). Increase in absorbance was measured at 405 nm for 10 min and plasmin activity was calculated as ΔmOD/min.

Detachment assay

BPAEC were seeded onto 24-well culture plates and grown to confluency. After washing with phosphate-buffered saline (PBS), FDPs were added in DMEM, containing 10% FCS. After 48 hours detached cells were removed and the remaining attached cells were counted after trypsinization. Percent endothelial cell detachment was calculated from the number of control cells – the number of treated cells / the number of control cells x 100%.

Tumor experiments

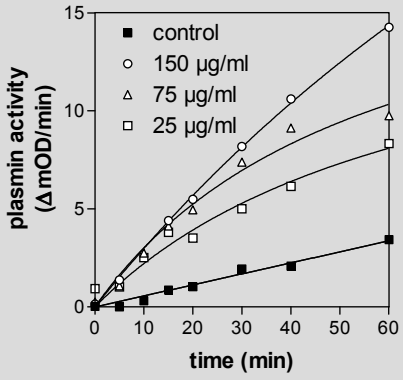
Male 6 to 8 week old BALB/c mice (General Animal Laboratory, University Medical Center Utrecht, The Netherlands) were used. All mice were fed a diet of animal chow and water *ad libitum*. Experiments were performed according to the guidelines of the Utrecht Animal Experimental Committee, University Medical Center (Utrecht, The Netherlands). C26 colon carcinoma cells were detached by brief trypsin exposure and after washing with PBS. $1x10^6$ C26 cells were inoculated subcutaneously. FDPs or control solvent was given by subcutaneous daily injection at a dose of 20 mg/kg/day. Tumor diameters were determined by calliper and volume was calculated with the formula: $width^2$ x length x 0.52.

Results

Fibrin degradation products (FDPs) were generated by cleaving polymerized fibrin with plasmin. FDPs stimulate tPA-mediated plasminogen activation (Figure 1). We investigated the effect of different concentrations of FDPs on confluent monolayers of bovine pulmonary artery endothelial cells (BPAEC). Treatment of endothelial cells with 1 µM FDPs for 48 hours did not cause morphological changes or cell detachment. However, incubation with 4 µM and 10 µM of FDPs resulted in 22.8 ± 0.2% and 33.7 ± 0.1% detachment respectively (Figure 2).

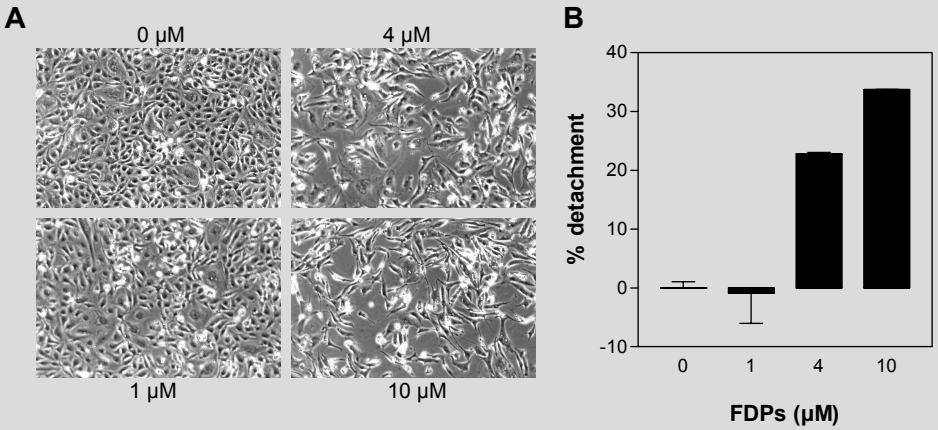
Antitumorigenic activity of FDPs was assessed in a subcutaneous tumor model. All mice had palpable tumors from day 4. Tumor volumes were followed every two days until tumor morbidity in the control group necessitated the termination of the experiment. Treatment with FDPs significantly reduced tumor growth. While tumors in control mice grew steadily over the whole treatment period to approximately 2.7 cm^3 at day 11 (Figure 3), treatment with FDPs (20 mg/kg/day) resulted in a significant inhibition of tumor growth during the injection period (75% from day 6). To establish the antitumorigenic potential of FDPs we compared tumor growth in mice treated with endostatin (10 mg/kg/day), an inhibitor of tumor growth that also stimulates tPA-mediated plasminogen activation[4]. Tumor growth inhibition by FDPs was similar to the effects elicited by endostatin.

Figure 1.



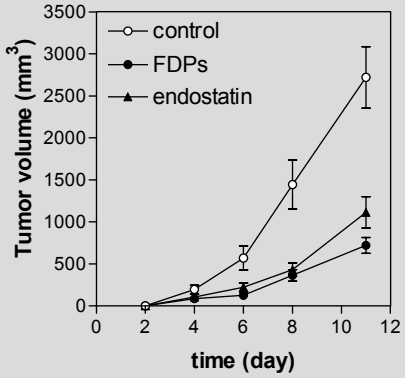
In vitro characterization of FDPs. Varying concentrations of plasmin-degraded fibrin were added to plasminogen and tPA. At several time points samples were taken and generated plasmin was analyzed using chromogenic substrate S-2251. Plasmin activity is shown as the rate of substrate conversion.

Figure 2.



FDPs cause endothelial cell detachment. (A) Bovine pulmonary artery endothelial cells were incubated with different concentrations of FDPs for 48 hours resulting in concentration-dependent cell detachment. (B) Endothelial cell detachment was quantified by calculating the number of control cells – number of treated cells divided by the number of control cells x 100.

Figure 3.



FDPs inhibit tumor growth. Mice (n=4/group) were inoculated subcutaneously with $1x10^6$ C26 colon carcinoma cells and treated daily with 500 µg FDPs, 500 µg endostatin or control solvent. Treatment with FDPs reduced tumor volume to 25 % of control at day 11. Values represent the mean volume ±SEM.

Discussion

We show here that plasmin-generated fragments of fibrin, FDPs, induce endothelial cell detachment and inhibit tumor growth.

Others have shown similar inhibitory effects using plasmin-derived fragments of fibrinogen. First, fibrinogen fragment D, which comprises the tPA-binding domain of plasmin-degraded fibrinogen, increases endothelial monolayer permeability[7] and causes detachment of bovine capillary endothelial cells[8]. These authors further show that cell detachment was due to the induced expression of tPA and uPA, subsequently leading to increased plasmin formation[8]. Second, plasmin-generated fibrinogen fragment E inhibits endothelial cell migration and tube formation *in vitro* and tumor growth in mice[9;10]. We used plasmin generated fragments of polymerized fibrin, which are known to circulate in cancer patients, and show that these fragments are deleterious for endothelial cells *in vitro*. Moreover and for the first time, we show that these tPA-stimulating fragments of fibrin inhibit tumor growth in mice.

Our data add FDPs to a substantial list of proteolytic fragments or denatured derivatives of plasma components that inhibit tumor growth (chapter 9). Little is known about the molecular mechanism(s) by which these endogenous proteins exert their actions. It has been shown that denatured proteins, either induced by proteolytic cleavage or denaturing agents, gain tPA-binding properties[11-14]. We noticed that many endogenous angiogenesis inhibitors, including endostatin[3], have the property to stimulate tPA-mediated plasminogen activation. This implicates that this type of angiogenesis inhibitors may share a common pathway that is induced by tPA binding proteins (see also chapter 9).

May activation of tPA reduce tumor growth? tPA is expressed by endothelial cells upon stimulation by angiogenic molecules like VEGF[15;16], tumor necrosis factor, epidermal growth factor[17;18], basic fibroblast growth factor[19] and thrombin[20]. Numerous studies revealed high expression of tPA in several human tumors[21-25] and clinical studies indicate that high tPA levels are associated with good prognosis in cancer patients[26-28]. Interestingly, fibrinolytic therapy using tPA potentially inhibits pulmonary seeding of intravenously injected mammary carcinoma cells in rats[29;30]. Others have shown that excessive plasminogen activation blocks angiogenesis in mice. Complete inhibition of tumor-induced angiogenesis in PAI-1 deficient mice was due to the uncontrolled elevation of plasmin activity[31]. In addition, treatment with pentoxifylline, an agent that releases tPA, inhibited human tumor implant-induced angiogenesis[32]. Our results further support the hypothesis that excessive plasminogen activation can lead to inhibition of angiogenesis and tumor growth.

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Chapter 9

Hypothesis: Do antiangiogenic protein fragments have amyloid properties?

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Abstract

Tumor growth requires proteolytic activity. As a consequence, protein breakdown products are present in the circulation of cancer patients. Within the past decade a large number of proteolytic fragments have been identified that inhibit angiogenesis and tumor growth. The mechanism(s) of action of these inhibitors is still poorly understood. We recently found that the effects of the angiogenesis inhibitor endostatin on endothelial cells is critically dependent on the presence of cross-β structure, a structure also present in amyloidogenic polypeptides in plaques of patients with amyloidosis, such as Alzheimer's disease. We also showed that cross-β structure containing endostatin is a ligand for tissue-type plasminogen activator (tPA). We noted that many angiogenesis inhibitors stimulate tPA-mediated plasminogen activation. Since the presence of cross-β structure is the common denominator in tPA-binding ligands, we hypothesize that these endogenous antiangiogenic proteolytic fragments share features with amyloidogenic polypeptides. We postulate that the cross-β structural fold, is present in these antiangiogenic polypeptide fragments and that this structure mediates the inhibitory effects. The hypothesis provides new insights in the potential mechanisms of antiangiogenesis and offers opportunities to improve the use of these inhibitory fragments.

Background

Angiogenesis, the formation of new blood vessels out of pre-existing vasculature, occurs in physiological and pathological processes, including embryonic development, the menstrual cycle, wound healing, inflammation and tumor growth (for review see[1]). In 1971, Folkman postulated that tumor growth is dependent on angiogenesis[2]. Accumulating evidence indicates that drugs that target the growing vasculature are promising new therapeutics for the treatment of cancer. Based on the concept that a primary tumor produces inhibitors of angiogenesis that can inhibit the outgrowth of metastases, the first endogenous angiogenesis inhibitor, termed angiostatin, was purified in the laboratory of Folkman in 1994[3]. The identification of angiostatin prompted the search for other angiogenesis inhibitors, resulting in the isolation of endostatin[4], prothrombin fragment 1 and 2[5], cleaved antithrombin III[6], fibrin(ogen) fragments[7] (and AR unpublished) and many other inhibitory polypeptide fragments. Commonly, such antiangiogenic polypeptides are proteolytically cleaved or denatured derivates of endogenous proteins. Endostatin, angiostatin and a thrombospondin peptide are currently being tested in clinical trials[8-10].

In 1997, Folkman and colleagues reported that treatment of experimental tumors with endostatin induced complete regression[11]. These magnificent results from animal studies fueled the great expectations that already accompanied the proposed use of antiangiogenic therapy with endogenous inhibitors of angiogenesis. Nobel laureate Watson was quoted as saying that "Judah is going to cure cancer in two years" and publications in the media did increase the hope for cure by many cancer patients. However, the initial results could not be reproduced by others and diminished the initial enthousiasm[12]. In fact, controversies regarding the antitumor effects of endostatin arose and initiated a heated discussion about its efficacy[12]. The variability in endostatin bioactivity is still unexplained. Originally, endostatin was purified from tissue culture supernatant. In the *in vivo* experiments that showed dramatic tumor regression a recombinant denatured and insoluble form of endostatin produced in Escherichia *coli* was used[11]. In a number of subsequent studies endostatin has been used from a number of different sources that caused less dramatic, minor or even no effects[13-16]. For studies in patients, a soluble globular form is used, produced in Pichia *pastoris* (http://entremed.com/pdfs/endostatin_factsheet.pdf). However, this globular form has no or very little effect on endothelial cells[17]. We found that only denatured endostatin is toxic to cells[18]. We wondered how our findings could explain the observed variability in endostatin bioactivity and to what extent our observations are of value for the use of other angiogenesis inhibitors.

Inherent toxicity of unfolded proteins

Similar to certain antiangiogenic polypeptides, toxic polypeptides involved in conformational diseases like Alzheimer's disease (AD), light-chain amyloidosis, pancreatic islet amyloidosis and spongiform encephalopathies are also proteolytic fragments or conformationally changed forms of proteins. Just recently a common mechanisms has been implied for such protein misfolding diseases. It was shown that toxicity is an inherent property of aggregates

of denatured proteins and is not related to the amino acid sequence composition of the protein[19]. Ultimately, structurally altered proteins are deposited as insoluble fibrillar aggregates and form plaques. Fibrillar aggregates are classified as amyloid fibrils based on the presence of cross-β structure. Cross-β structures in amyloid fibrils are stacked β-sheets likely composed of flat and non-twisted β-strands. This will result in a unique and flat 2D β-sheet surface, not seen in globular proteins. Many studies have indicated a close relationship between the presence of amyloid depositions and vascular damage. First, transmissible spongiform disease is associated with the presence of the abnormal form of prion protein that aggregates into amyloid fibrils. These prion depositions are toxic to a wide variety of cells, including cerebral endothelial cells[20]. In addition to neuronal degeneration, AD patients exhibit significant cerebrovascular pathology[21;22]. Furthermore, it is known that patients with AD often suffer from stroke and that hemorrhages are frequently present[23]. *In vitro* experiments revealed that amyloid β increases permeability of endothelial cell monolayers and induces apoptosis[24;25]. Other studies have shown that smooth muscle cells and endothelial cells are damaged in cerebral blood vessels of AD cases[25-27]. We recently showed that insoluble endostatin forms amyloid fibrils, suggesting that endostatin may induce apoptosis as a consequence of its amyloid structure[18;28]. Interestingly, in AD

Table. Overview of antitumorigenic proteins with tPA stimulating activity.

	Effect on angiogenesis or tumor growth	Stimulation of tPA-mediated plasminogen activation
Endostatin	O'Reilly et al., 1997	Reijerkerk et al., 2003
Thrombospondin[1]	Volpert et al., 1998	Silverstein et al., 1985; Silverstein et al., 1984; Silverstein et al., 1986
Angiostatin[2]	O'Reilly et al., 1994	unknown
Denatured ATIII	O'Reilly et al., 1999	Machovich and Owen, 1997
Prothrombin fragments	Rhim et al., 1998	Machovich et al., 1999
βpep25 (Anginex)	Griffioen et al., 2001	Reijerkerk, unpublished
Maspin	Zou et al., 1994	Sheng et al., 1998
Histidine-proline-rich glycoprotein	Juarez et al., 2002	Silverstein et al., 1985; Borza and Morgan, 1997
Fibrin(ogen) degradation products	Chapter 8 and Brown et al., 2002	Stewart et al., 1998
Calreticulin (fragments), vasostatin	Pike et al., 1999	binds tPA, Allen and Bulleid, 1997
Amphoterin[3]	Huttunen et al., 2002	Parkkinen and Rauvala, 1991

[1] Thrombospondin also binds the multiligand cross-β structure receptor CD36[35;52].

[2] Angiostatin that is tested in clinical trials is made in Pichia *pastoris*. The original experiments however were performed with angiostatin prepared by proteolytic cleavage with elastase, followed by purification on a lysine-sepharose column and subsequent dialysis against H_2O[3]. The latter method of purification leaves the possibility that fragments are present with amyloid properties. We therefore suggest that different forms of angiostatin may also yield differences in bioactivity.

[3] Amphoterin also binds the multiligand cross-β structure receptor RAGE[34].

endostatin colocalized with amyloid-β positive plaques that were surrounded by focal gliosis[29]. Is the cross-β structure a candidate common denominator among antiangiogenic polypeptide fragments?

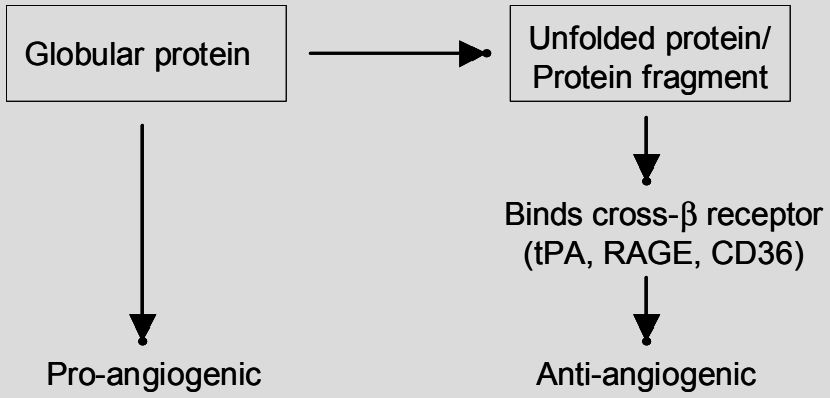
The tPA connection

It is established that denatured proteins can stimulate plasminogen activation by the serine protease tissue-type plasminogen activator (tPA)[30]. Amyloid fibrils such as amyloid β and prion protein markedly stimulate plasminogen activation by t-PA[31;32]. We found that the presence of cross-β structure is a prerequisite for tPA binding to and stimulation of tPA-mediated plasminogen activation by fibrinogen fragments, islet amyloid polypeptide (IAPP) and endostatin[28]. Moreover, this structure mediated tPA-dependent endostatin-induced cell detachment[17]. Activation of tPA by amyloid polypeptides may induce excessive degradation of extracellular matrix with subsequent loss of cell attachment sites resulting in cell detachment and apoptosis[17;33]. We noticed that many antiangiogenic compounds, generated through proteolytical cleavage or denaturation, similarly stimulate tPA-mediated plasminogen activation (Table).

Hypothesis

It is intriguing that unrelated proteins lacking sequence homology acquire antiangiogenic activity upon proteolytic cleavage or denaturation and at the same time acquire the ability to stimulate tPA-mediated plasminogen activation. Toxicity of aggregates of denatured proteins is also independent of the amino acid sequence[19]. Taken together, this suggests that a common antiangiogenic pathway may exist that is induced by tPA binding proteins. Thus, we hypothesize that antiangiogenic polypeptide fragments contain the cross-β structure and

Figure.



Schematic representation of the hypothesis.

that this structure is the common denominator responsible for their antiangiogenic and antitumor effects. If this hypothesis is correct, other multiligand cross-β structure receptors in addition to tPA may also mediate the antiangiogenic response. There is good evidence that at least two other multiligand receptors, CD36 and the receptor for advanced glycation end-products (RAGE) are involved[34;35]. A schematic presentation of our hypothesis is illustrated in the figure.

Implications

If correct, our hypothesis implies that (i) it is relevant to look at structural properties of these polypeptide inhibitors, rather than at their primary amino acid sequence and consider the possibility that an active drug requires a cross-β structure. (ii) That the inhibitory effect of the drugs listed in Table may be improved by adding factors that are known to increase amyloid-mediated toxicity, such as LPS, TNFα and IFNγ[36;37]. (iii) It is necessary to use these polypeptide fragments with caution as they have a high probability to form toxic aggregates.

If our hypothesis is correct tumor derived endogenous circulating inhibitors may have antiangiogenic their activity also as consequence of the cross-β structure. Besides their antiangiogenic activity, these circulating proteolytic fragments may however then also contribute to some of the clinical complications of cancer, such as amyloidosis or bleeding events. Such circulating unfolded polypeptide fragments with amyloid properties may help to explain the bad clinical condition of cancer patients with advanced disease.

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Chapter 10

Summary and general discussion

The hemostatic system serves to maintain normal blood flow and protects us against blood loss after injury. Upon vessel injury the coagulation cascade is activated and initiates the formation of a thrombus, consisting of fibrin and activated platelets. This clot provides a provisional barrier that occludes the vessel. Subsequent activation of the fibrinolytic cascade is needed for clot lysis and repair of injury. Tissue repair is dependent on the formation of new blood vessels, a process called angiogenesis. Angiogenesis is not only necessary for tissue repair. In 1971, Folkman postulated that angiogenesis is also important for tumor growth. It is now well established that the hemostatic system controls angiogenesis and tumor growth. Fibrin is present in tumors and facilitates angiogenesis by supporting endothelial and tumor cell attachment, migration and proliferation. Moreover, fibrin is important for metastasis of circulating tumor cells. Studies in mice lacking components of the fibrinolytic system, including plasminogen, plasminogen activator and plasminogen activator inhibitor-1 (PAI-1) have implicated a role of these proteins in angiogenesis and tumor development and metastasis. Markers of active fibrinolysis and coagulation circulate in blood of cancer patients. Interestingly, breakdown products of circulating proteins of the hemostatic system such as angiostatin (a fragment of plasminogen), prothrombin fragment 1 and 2 and antiangiogenic antithrombin III (aaATIII) can inhibit tumor growth.

The subject of this thesis focuses on the role of the plasminogen activation system in cancer. The plasminogen activation system regulates fibrinolysis and controls cell migration and invasion by plasmin-mediated matrix proteolysis. Plasmin is formed upon cleavage of the zymogen plasminogen by plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). In contrast to uPA, plasminogen activation by tPA requires a cofactor. Fibrin and degraded fibrin are well known cofactors of tPA-mediated plasminogen activation. Plasmin cleaves its substrates behind a basic amino acid (lysine or arginine). Free carboxy-terminal lysines or arginines provide high affinity binding sites for plasminogen and facilitate efficient activation by plasminogen activators. This subsequently leads to the formation of more plasminogen binding sites resulting in an amplification loop for plasminogen activation. Besides fibrin, a variety of cell types also have carboxy-terminal lysine-dependent binding sites for plasminogen that can mediate plasminogen activation. Plasminogen activation can be inhibited by lysine analogues such as ε-aminocaproic acid (εACA) or tranexamic acid. Studies with these lysine analogues have indicated the importance of carboxy-terminal lysine residues in cancer and angiogenesis.
Thrombin-activatable fibrinolysis inhibitor (TAFI, carboxypeptidase U, R or plasma procarboxypeptidase B) is made by the liver and circulates in blood in its inactive form. Active TAFI was first isolated from fresh serum in 1990. When converted into its active form by thrombin, plasmin or trypsin, TAFI is a carboxypeptidase B-type enzyme that specifically cleaves carboxy-terminal lysine or arginine residues from proteins. Removal of carboxy-terminal lysines and arginines from fibrin abrogates efficient plasminogen binding and consequently leads to decreased plasmin formation. Experiments in mice have indicated that TAFI is a regulator of fibrinolysis *in vivo*. Thrombin-mediated activation of TAFI is

stimulated 1250-fold by the endothelial cell receptor thrombomodulin which is expressed on endothelial cells and in tumors. We tested the hypothesis that TAFI controls tumor growth.

In **chapter 1** we give a literature overview of the plasminogen activation system in cancer. In addition we summarize possible ways to interfere with tumor growth by targeting this system. We conclude that proper angiogenesis and tumor growth depends on a perfect balance of coagulation and fibrinolysis. Consequently, disturbance of this balance, either by inhibition, but also by overstimulation of the fibrinolytic system might inhibit angiogenesis and tumor growth. We postulate that the development of molecules that lead to excessive proteolysis in the tumor may be a powerful antiangiogenic and antitumorigenic approach.
To clarify the role of proteins involved in the regulation of fibrinolysis during angiogenesis, we have studied corneal vessel formation in mice deficient for urokinase-type plasminogen activator (uPA), tissue-type plasminogen activator (tPA), plasminogen, plasminogen activator inhibitor-1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI) **(chapter 2)**. Our results corroborate earlier findings that angiogenesis in the mouse cornea is dependent on PAI-1 and, albeit minoraly, plasminogen. Although it is widely accepted that plasminogen is an important mediator of angiogenesis, corneal angiogenesis can occur in the absence of plasminogen. Others reported the involvement of membrane-type matrix metalloproteinases that are essential in this process. Our results suggest the presence of plasmin-independent mechanisms that can activate these proteases during corneal angiogenesis. Interestingly, in previous studies, we have shown that corneal neovascularization was almost completely inhibited by continuous treatment with angiostatin, a fragment of plasminogen. The results described in this thesis suggest that the antiangiogenic effect of angiostatin *in vivo* may not only be mediated through inhibition of plasminogen function. The absence of tPA, uPA or TAFI did not affect the formation of new vessels in the cornea. Others have shown that uPA antagonists can inhibit corneal neovascularization in rabbits and angiogenesis in mice. Our results therefore indicate that the antiangiogenic effects of uPA inhibitors *in vivo* may be largely due to other activities than direct uPA inhibition.
Given the importance of the plasminogen activation system in tumor growth and metastasis, we next investigated whether TAFI plays a regulatory role in these processes. We analyzed subcutaneous tumor growth and experimental- and spontaneous metastasis formation in TAFI-deficient mice **(chapter 3)**. Conceivably, disturbance of tightly controlled plasminogen activation in TAFI-deficient mice might cause decreased primary tumor growth and tumor cell migration resulting in the formation of less experimental and spontaneous metastasis. On the other hand, increased plasminogen activation in the absence of TAFI could cause increased matrix degradation in the primary tumor resulting in increased tumor growth, efficient entrance of tumor cells into the circulation and elevated spontaneous metastasis formation. However, none of these possible effects were seen. We suggest two potential explanations for the lack of differences in tumor growth and metastasis in TAFI-deficient mice. First, TAFI-deficiency does not result in a significant change in plasmin formation due to the presence of other regulators that neutralize the effect of TAFI-deficiency on plasmin

activation or activity. An alternative explanation for the lack of effect on tumor growth is that excess plasmin in TAFI-deficient mice has no effect at all in the models used. A second explanation for our observations is that TAFI is not activated at all and does not regulate plasminogen activation in cancer and metastasis. Unfortunately, at present we cannot determine whether TAFI is activated in cancer.
Furthermore, we have determined TAFI antigen and TAFI activity levels in patients with primary and metastasized prostate cancer **(chapter 4)**. It is well known that the fibrinolytic and coagulation systems are activated in (metastatic) prostate cancer. In these patients TAFI could be activated and consumed by the coagulation (thrombin) and/or fibrinolytic (plasmin) system or either inactivated by increased plasmin activity in prostate cancer patients. However, we did not observe abnormal TAFI antigen or activity levels in these patients. In addition, TAFI antigen or activity was not associated with cancer progression and the incidence of thrombosis **(chapter 4)**.

The involvement of hemostatic system components in angiogenesis and cancer offers new possibilities for cancer treatment. Intriguingly, several antiangiogenic (cryptic) fragments of proteins regulating hemostasis, such as angiostatin and antiangiogenic antithrombin III (aaATIII), have been identified during the past decade. Our main challenge was to elucidate the mechanism of action of endostatin **(chapter 5)**. Endostatin is a carboxy-terminal fragment of collagen XIII, and has originally been described as one of the most potent inhibitors of angiogenesis. In animal experiments different forms of endostatin are used. Although the soluble form of endostatin inhibits tumor growth, complete tumor regression has only been observed when using bacterially produced insoluble endostatin. Likely, these forms have different bioactivities. We found that insoluble endostatin, in contrast to soluble endostatin, stimulates plasminogen activation by tPA, induces plasminogen-mediated endothelial cell detachment and matrix degradation and is toxic to neuronal cells **(chapter 5,6)**. Endostatin-induced plasminogen activation, matrix degradation and endothelial cell detachment were inhibited by carboxypeptidase B treatment indicating an essential role for carboxy-terminal lysines. In line with this, cotreatment of mice with insoluble endostatin and carboxypeptidase B abolished the inhibitory effect of endostatin. Similarly, endostatin-mediated stimulation of plasminogen activation by tPA was inhibited by the physiological regulator of plasminogen activation in plasma, TAFI. Thus in principle, TAFI could function as a regulator of the antitumor activity of endostatin.
Whereas the structure of soluble, globular endostatin has been elucidated, the structure of insoluble endostatin has not. Insoluble proteins may occur as amorphous aggregates or as highly ordered ‘amyloid’ deposits in which the polypeptide backbones are stacked through intermolecular hydrogen bonds, forming a cross-β sheet. The cross-β structure is a hallmark of proteins or protein fragments in amyloid plaques of patients with amyloidosis, such as Alzheimer’s disease. Structure analyses revealed that insoluble endostatin forms amyloid fibers through extensive of cross-β structure formation **(chapter 6)**. Interestingly, the effects of insoluble endostatin on plasminogen activation and endothelial and neuronal cells were

critically dependent on the presence of this structure **(chapter 5,6,7)**. Since it is well established that there is great variability among preparations of amyloid aggregates and the resulting toxicity, our finding may explain some of the large differences in the results that have been obtained with various sources of endostatin.

The above results prompted us to investigate the binding characteristics of several other amyloid peptides to tPA. We established that the cross-β structure is a common feature in other tPA-ligands, including fibrin peptides, islet amyloid polypeptide (IAPP, associated with pancreatic β cell toxicity in type II diabetes) and amyloid β (associated with Alzheimer's disease), and responsible for their ability to stimulate tPA-mediated plasminogen activation **(chapter 7)**. Our results have classified tPA as a multiligand cross-β structure binding protein. tPA may prevent the accumulation of toxic (improperly folded) proteins in the circulation by inducing plasmin-mediated proteolysis. Alternatively, tPA may mediate cross-β structure induced cell toxicity via excessive matrix degradation. tPA has already been implicated in smooth muscle cell apoptosis, neuronal cell toxicity and endothelial cell detachment. Thus, excessive formation of plasmin through activation of tPA may inhibit tumor growth.

Fibrin degradation products (FDPs) also stimulate plasminogen activation through tPA. Moreover, FDPs circulate in blood of cancer patients. We tested the hypothesis that fibrin fragments also can induce endothelial cell detachment and inhibit tumor growth **(chapter 8)**. We found that fibrin degradation products (FDPs) induce detachment of bovine pulmonary endothelial cells and inhibit the growth of subcutaneous tumors in mice. These results further strengthen the idea that tPA-activating proteolytic fragments in general can inhibit angiogenesis and tumor growth.

To what extent may our observations be of value for the use of endostatin and other antiangiogenic fragments? In **chapter 9** we discuss the possible implications of our findings. We found that endostatin can stimulate plasminogen activation by the serine protease tissue-type plasminogen activator (tPA). We noticed that many antiangiogenic compounds, generated through proteolytical cleavage or denaturation stimulate tPA-mediated plasminogen activation. It is intriguing that unrelated proteins lacking sequence homology acquire antiangiogenic activity upon proteolytic cleavage or denaturation and at the same time acquire the ability to stimulate tPA-mediated plasminogen activation. Taken together, this suggests that a common antiangiogenic pathway may exist that is induced by tPA binding proteins. We hypothesize that antiangiogenic polypeptide fragments contain the cross-β structure and that this structure is the common denominator responsible for their antiangiogenic and antitumorigenic effects. Our results imply that it is relevant to consider the possibility that an active drug requires the formation of cross-β structure. However, a consequence of our hypothesis is that it is necessary to be cautious to use polypeptide fragments as they can have a high probability to form toxic aggregates. A final and intriguing implication of our hypothesis is that the presence of the cross-β structure in tumor-derived endogenous circulating inhibitors may be responsible for the antiangiogenic activity. Besides their antiangiogenic activity, these fragments may also contribute to some of the clinical complications, such as amyloidosis or bleeding events, which are observed in cancer patients with advanced disease.

Samenvatting in het Nederlands

In mens en dier verzorgen bloed en bloedvaten de noodzakelijk aanvoer van zuurstof en voedsel naar de verschillende organen. Bij een verwonding worden bloedvaten beschadigd. Om te voorkomen dat er teveel bloedverlies optreedt, wordt er een bloedstolsel gevormd. Een complex systeem van eiwitten reguleert de vorming en de afbraak van een stolsel. Het systeem dat de vorming van een stolsel induceert wordt het stollingssysteem genoemd. Het systeem dat zorgt voor de afbraak van het stolsel heet het fibrinolytische systeem. Het stollingssysteem en het fibrinolytische systeem zorgen samen voor het in stand houden van een goede doorbloeding. Dit wordt hemostase genoemd.
Een bloedstolsel ontstaat door samenklontering van bloedeiwitten en geactiveerde bloedplaatjes. Een stolsel voorkomt niet alleen verder bloedverlies, maar de in het stolsel aanwezige eiwitten en bloedplaatjes zorgen ook voor het herstel van het beschadigde weefsel. In eerste instantie herstellen ze de bloedvoorziening door de vorming van nieuwe bloedvaatjes te stimuleren. De vorming van nieuwe bloedvaatjes wordt angiogenese genoemd. Tijdens wondgenezing speelt het fibrinolytische systeem een cruciale rol. Het fibrinolytische systeem bestaat uit een cascade van enzymen (het plasminogeenactivatie systeem) dat zorgt voor de afbraak van het stolsel.
De vorming van nieuwe bloedvaten (angiogenese) is niet alleen betrokken bij herstel van weefselschade. In 1971 poneerde Judah Folkman de hypothese dat angiogenese ook belangrijk is voor de groei van tumoren. Als tumoren groter worden dan enkele mm^3 is de voedselvoorziening via diffusie niet meer toereikend en dient het bloedvatstelsel uitgebreid te worden. Uitgebreid onderzoek naar de betrokkenheid van het hemostase systeem bij angiogenese en kanker heeft inmiddels een duidelijke rol voor dit systeem in deze processen aangetoond. Stollingseiwitten zijn aanwezig in tumoren en faciliteren de hechting en migratie van cellen die de bloedvatwand bekleden (endotheelcellen) en van tumorcellen. Daarnaast is bekend dat stollingseiwitten een belangrijke rol spelen bij de uitzaaiing van tumoren. Verscheidene studies hebben aangetoond dat het fibrinolytische systeem betrokken is bij angiogenese, bij tumorgroei en bij het ontstaan van uitzaaiingen. Op basis van metingen van stollingseiwiten en fibrinolytsche parameters kunnen we concluderen dat het hemostase systeem geactiveerd is in kankerpatiënten.

In dit proefschrift worden de resultaten beschreven van het onderzoek naar de rol van het fibrinolytische systeem bij de vorming van nieuwe bloedvaten in kanker. Bij kanker reguleert het fibrinolytische systeem de afbraak van extracellulaire matrix (opgebouwd uit componenten van een stolsel). De afbraak van deze matrix is noodzakelijk voor de groei en invasie van endotheel- en tumorcellen. Plasmine is één van de belangrijke enzymen voor de afbraak van extracellulaire matrix en wordt gevormd na activatie van het bloedeiwit plasminogeen. Twee enzymen kunnen voor de activatie van plasminogeen zorgen, tPA en uPA. tPA-gemedieerde plasminogeenactivatie neemt sterk toe in de aanwezigheid van een cofactor. De cofactor is vaak een afgebroken onderdeel van de extracellulaire matrix. De

cofactor bind zowel tPA als plasminogeen en zorgt ervoor dat deze eiwitten in de positie komen die nodig is voor optimale activatie van plasminogeen. Plasmine knipt de eiwitten van de extracellulaire matrix specifiek na een lysine of arginine. Daardoor ontstaan er fragmenten met aan het eind een lysine of arginine. Deze eindstandige aminozuren fungeren als bindingsplaats voor plasminogeen en zijn belangrijk voor tPA-gemedieerde activatie van plasminogeen. Omdat meer plasmine resulteert in de vorming van meer eindstandige lysines of arginines die weer zorgen voor een toename in plasminogeen binding en activatie, ontstaat er een waterval effect. Plasminogeenactivatie wordt geremd door moleculen die lijken op lysine. Cyclokapron is een voorbeeld van een lysine-analoog dat daarom wordt gebruikt om bij bepaalde patiënten bloedingen te helpen voorkomen. Studies met dergelijke middelen hebben aangetoond dat eindstandige lysines belangrijk zijn bij angiogenese en kanker.
Trombine-activeerbare fibrinolyse inhibitor (TAFI) wordt gemaakt door de lever en circuleert in bloed in een inactieve vorm. TAFI wordt geactiveerd door de enzymen trombine, plasmine en trypsine. Trombine-gemedieerde TAFI activatie neemt 1250 maal toe in aanwezigheid van trombomoduline, een eiwit dat aanwezig is op endotheel- en tumorcellen. Actief TAFI is een carboxypeptidase B-type enzym en verwijdert eindstandige lysines of arginines van eiwitten. Verwijdering van deze plasminogeen bindingsplaatsen in eiwitten resulteert in de afname van plasminogeenactivatie en fibrinolyse. Wij hebben uitgezocht of TAFI een rol kan spelen bij kanker.

In **hoofdstuk 1** wordt een overzicht gegeven van de huidige stand van zaken met betrekking tot het onderzoek naar de rol van het plasminogeenactivatie systeem bij kanker. Daarnaast zijn een aantal benaderingen samengevat die tot doel hebben dit systeem te gebruiken als aangrijpingspunt voor antitumor therapie. We concluderen dat angiogenese en tumorgroei afhankelijk is van een juiste afstemming van stolling en fibrinolyse. Dit betekent dat interventies die dit evenwicht opheffen, zoals remming, maar ook overactivering van het fibrinolytische systeem, leiden tot verminderde angiogenese en tumorgroei. We postuleren dat moleculen die overmatige fibrinolyse in de tumor induceren potentiële kandidaten zijn voor de behandeling van kanker.
Om meer duidelijkheid te krijgen over de rol van het plasminogeenactivatie systeem in angiogenese hebben we gebruik gemaakt van een angiogenesemodel in het hoornvlies (cornea) van muizen deficiënt voor uPA, tPA, plasminogeen, PAI (een remmer van plasminogeenactivatie) en TAFI. **In hoofdstuk 2** laten we zien dat angiogenese in de cornea van muizen sterk afhankelijk is van de aanwezigheid van PAI en in veel mindere mate van plasminogeen. In het verleden hebben we aangetoond dat angiostatine, een fragment van plasminogeen, de corneale angiogenese compleet kan remmen. Onze bevindingen suggereren dat het antiangiogene effect van angiostatine in muizen niet alleen bepaald wordt door remming van plasminogeen. De afwezigheid van plasminogeen-activatoren, tPA en uPA was niet van invloed op de vorming van nieuwe bloedvaten in de cornea. Ook TAFI was niet van invloed op de vorming van bloedvaten in de cornea. Het feit

dat anderen hebben laten zien dat corneale angiogenese effectief geremd wordt door uPA-antagonisten is opmerkelijk, en suggereert dat deze effecten bepaald worden door een activiteit anders dan uPA-remming.
Omdat het plasminogeenactivatie systeem betrokken is bij tumorgroei en het ontstaan van uitzaaiingen, hebben we onderzocht of TAFI deze processen reguleert. Voor dit onderzoek hebben we tumorgroei van onderhuidse (subcutane) tumoren en de vorming van uitzaaiingen bestudeerd in muizen die geen TAFI maken **(hoofdstuk 3)**. Het is denkbaar dat in de afwezigheid van TAFI, plasminogeenactivatie zodanig gedereguleerd wordt dat er overmatige matrixafbraak plaatsvindt, resulterend in verminderde tumorgroei en verminderde vorming van uitzaaiingen (zie ook hoofdstuk 1). Anderzijds kan het zijn dat verhoogde plasminogeenactivatie leidt tot versnelde matrixafbraak, tumorgroei en de vorming van uitzaaiingen. Echter, geen van deze effecten zijn waargenomen. Er zijn verschillende verklaringen mogelijk. In de eerste plaats is het mogelijk dat door de aanwezigheid van andere remmers van plasminogeenactivatie, bijvoorbeeld PAI, de afwezigheid van TAFI niet resulteert in een toename van plasmine. Een andere verklaring zou kunnen zijn dat overmatige plasminogeenactivatie geen effect heeft in onze modellen. In de tweede plaats zou het kunnen zijn dat TAFI niet geactiveerd wordt tijdens tumorgroei en de vorming van uitzaaiingen en dus ook niet plasminogeenactivatie reguleert.
Het is bekend dat het coagulatie- en fibrinolytische systeem geactiveerd is in patiënten met kanker. Wij hebben TAFI antigeen en activiteit bestudeerd in bloed van patiënten met primaire en uitgezaaide prostaatkanker. Het zou kunnen zijn dat TAFI geactiveerd en verbruikt wordt door het coagulatie- (trombine) of fibrinolytisch (plasmine) systeem of mogelijk geïnactiveerd door verhoogde plasmine-activiteit in deze patiënten. Echter, TAFI concentratie en activatie zijn normaal in bloed van patiënten met prostaatkanker. Ook zijn de gemeten waarden niet geassocieerd met de progressie van de ziekte of met trombotische complicaties.

De bevindingen uit het onderzoek naar de rol van het hemostase systeem in angiogenese en kanker hebben nieuwe mogelijkheden gegeven voor de ontwikkeling van nieuwe medicijnen voor de behandeling van kanker. In de afgelopen tien jaar zijn er verschillende angiogeneseremmers ontdekt. Het is opmerkelijk en interessant dat veel angiogeneseremmers proteolytische fragmenten zijn van eiwitten van het hemostase systeem. Ons onderzoek heeft zich gericht op de ontrafeling van het werkingsmechanisme van de angiogeneseremmer endostatine. Endostatine is een fragment van het extracellulaire matrixeiwit collageen XVIII en is in 1997 beschreven als de meest potente remmer van tumorgroei. Echter, het gebruik van verschillende vormen van endostatine in combinatie met uiteenlopende behandelingsresultaten heeft het onderzoek naar de werking van endostatine gecompliceerd. Alhoewel een oplosbare vorm van endostatine tumorgroei remt, is regressie van tumoren alleen waargenomen na behandeling met een gedenatureerde, onoplosbare vorm, gemaakt in bacteriën. Het is aannemelijk dat deze twee vormen een verschillende

biologische functie hebben. Wij hebben aangetoond dat de onoplosbare vorm van endostatine, in tegenstelling tot de oplosbare vorm, tPA-gemedieerde plasminogeenactivatie stimuleert. Endostatine-geïnduceerde plasminevorming resulteert in matrixafbraak en in het loslaten van endotheelcellen **(hoofdstuk 5,6)**. Stimulatie van plasminogeenactivatie, matrixafbraak en het loslaten van endotheelcellen wordt geremd door carboxypeptidase B. Dit impliceert dat carboxyterminale lysines een belangrijke rol spelen in deze endostatine-geïnduceerde processen. In overeenstemming hiermee is de waarneming dat gelijktijdige behandeling van experimentele tumoren met carboxypeptidase B de antitumorale effecten van onoplosbaar endostatine opheft. Endostatine-gestimuleerde plasminogeenactivatie wordt op een vergelijkbare manier geremd door TAFI, een fysiologische regulator van plasminogeenactivatie. Het is niet ondenkbaar dat TAFI de werking van endostatine in muizen reguleert.

We hebben de structuur van de onoplosbare vorm van endostatine opgehelderd. Het was bekend dat onoplosbare eiwitten voorkomen als amorfe aggregaten of als goed geordende fibrillen. In deze aggregaten en fibrillen zijn de polypeptiden met elkaar verbonden via waterstofbruggen en vormen ze een cross-β structuur. Deze structuur is een kenmerk van eiwitten of eiwitfragmenten zoals die worden gevonden in eiwitdeposities in patiënten met amyloidose. Bekende ziektes die gepaard gaan met de depositie van amyloid zijn de ziekte van Alzheimer en de gekke-koeienziekte. Door middel van gedetailleerde analyse hebben we aangetoond dat onoplosbaar endostatine ook cross-β structuren bevat en amyloid vormt **(hoofdstuk 6)**. De door ons beschreven effecten van deze vorm van endostatine op tPA-gemedieerde plasminogeenactivatie, op endotheelcellen en op neurale cellen was afhankelijk van de aanwezigheid van de cross-β structuur **(hoofdstuk 5,6,7)**. Het is bekend dat de mate van aggregatie en de toxiciteit van verschillende preparaten van een amyloid eiwit sterk kan variëren. Mogelijk leveren onze bevindingen een verklaring voor de verschillen in activiteit tussen afzonderlijke preparaten van endostatine.

De hiervoor beschreven resultaten hebben ons aangezet om de binding van tPA aan amyloide peptiden nader te bestuderen. We hebben aangetoond dat de cross-β structuur een algemeen voorkomende structuur is in fibrine peptiden, islet amyloid polypeptide (IAPP, geassocieerd met pancreas β cel toxiciteit in type II diabetes) and amyloid β (betrokken bij de ziekte van Alzheimer). Daarnaast is deze structuur verantwoordelijk voor de eigenschap van deze peptiden om tPA-gemedieerde plasminogeenactivatie te stimuleren **(hoofdstuk 7)**. Onze resultaten typeren tPA als een algemeen cross-β structuur bindend enzym. We veronderstellen dus dat tPA, via plasmine- gemedieerde eiwitafbraak een belangrijke functie heeft in het voorkomen van een ongewenste opstapeling van toxische (gedenatureerde, verkeerd gevouwen) eiwitten in de bloedvaten. Het is ook goed mogelijk dat tPA juist betrokken is bij amyloid-geïnduceerde celdood (apoptose) als gevolg van overmatige matrixafbraak. Het is al bekend dat tPA een rol speelt bij de dood van gladde spiercellen en neuronale cellen. Het zou dus goed mogelijk kunnen zijn dat overmatige plasminevorming en matrixafbraak leidt tot celdood en remming van tumorgroei.

Afbraakproducten van fibrine, de belangrijkste component van een bloedstolsel, komen in verhoogde concentraties voor in bloed van kankerpatiënten, en kunnen ook plasminogeenactivatie door tPA stimuleren. Wij hebben getest of afbraakproducten van fibrine een effect hebben op de hechting van endotheelcellen en op de groei van kanker **(hoofdstuk 8)**. Onze resultaten laten zien dat behandeling van endotheelcellen met afbraakproducten van fibrine het loslaten van deze cellen induceert. Daarnaast vonden we dat deze fragmenten, vergelijkbaar met endostatine, subcutane tumorgroei in muizen remmen. Deze resultaten vormen een nieuwe aanwijzing dat tPA-activerende proteolytische fragmenten in het algemeen angiogenese en tumorgroei kunnen remmen.

In hoeverre zijn onze bevindingen van belang voor het gebruik van endostatine en andere antiangiogene fragmenten? In **hoofdstuk 9** worden de mogelijke implicaties van onze resultaten bediscussieerd. Samengevat constateren we het volgende. De angiogenese- en tumorgroeiremmer endostatine stimuleert tPA-gemedieerde plasminogeenactivatie. Daarnaast weten we dat een aantal andere antiangiogene middelen, als proteolytisch fragment of gedenatureerd eiwit, ook plasminogeenactivatie door tPA stimuleren. Interessant is dat verschillende eiwitten (zonder homologie van aminozuur sequentie) antiangiogeen/antitumorigeen zijn na proteolyse of denaturatie en tegelijkertijd de potentie verwerven tPA-gemedieerde plasminogeenactivatie te stimuleren. Dit suggereert de aanwezigheid van een gemeenschappelijk antiangiogeen mechanisme dat wordt geactiveerd door tPA-bindende peptiden. Wij postuleren dat antiangiogene eiwitfragmenten de cross-β structuur bevatten en dat deze structuur de antiangiogene en antitumorigene activiteit bepaald. Onze resultaten impliceren verder dat de aanwezigheid van deze structuur niet onbelangrijk is voor de effectiviteit van antiangiogene polypeptide fragmenten. Aan de andere kant is de consequentie van onze hypothese dat we voorzichtig moeten zijn met het gebruik van dit type middelen in verband met mogelijke amyloid-gerelateerde toxiciteit op langere termijn. Naast de antiangiogene activiteit van circulerende eiwitfragmenten kan het ook zijn dat deze fragmenten bijdragen aan sommige klinische complicaties, zoals amyloidose en bloedingen, die waargenomen worden in patiënten met een ver gevorderd stadium van kanker.

Dankwoord

In dit waarschijnlijk meest gelezen gedeelte van mijn proefschrift wil ik graag mijn dank uitspreken voor de bijdragen van velen die de totstandkoming ervan mogelijk maakten. Tegelijkertijd loop ik een groot risico mensen te vergeten. Omdat ik mij hiervan bewust ben, wil ik ook iedereen die vindt dat zijn hulp ten onrechte niet genoemd is, hoewel daar alle reden voor bestaat, mijn hartelijke dank betuigen.

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Co-promotor, Dr. M.F.B.G. Gebbink. Beste Martijn, het zit erop. Ik ben je erg dankbaar voor je intensieve begeleiding. Het gezamenlijk schrijven van het review voor The European Journal of Cancer met de daarbij behorende oeverloze discussies op toen nog ‘onze kamer’ heeft mij op een snelle manier veel theoretische kennis opgeleverd. Veel heb ik geleerd van je brede interesse voor de wetenschap en niet-aflatende drang bestaande wetenschappelijke kennis maximaal te integreren in ons eigen onderzoek. Promoveren is ploeteren, vallen, opstaan. Ik beken je dat ik niet zelden het idee had dat het niet goed zou komen. Ons probleem (endostatine) leek niet oplosbaar. Achteraf bleek daar juist de oplossing te liggen wat aanleiding gaf tot een drietal publicaties. Ik denk met veel plezier aan onze samenwerking terug.

Collega’s van het Laboratorium Experimentele Oncologie. Werken op het lab heb ik dankzij jullie en de prettige, gezellige en saamhorige sfeer, altijd als zeer plezierig ervaren. Ik bedank allen die mij met raad en daad hebben bijgestaan.

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met geperforeerd hoornvlies, zou ik je hulp niet nodig gehad hebben, wat ben ik toch onhandig) en Lisette te Velde, bedankt voor jullie hulp met de *in vivo* experimenten.

Colinda Aarsman, duizendpoot, je stond altijd klaar om me te helpen. Ik weet niet of ik me moet excuseren, misschien had je gewoon een betere verdedigingswal moeten aanleggen op de grens tussen onze werkplekken. Cor Seinen, Bettina Brünjes en Agnes Burema, bedankt voor jullie bijdrage aan het tPA-onderzoek. Ik vond het plezierig om met jullie samen te werken.

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Publicaties

Reijerkerk, A., Voest, E. E., and Gebbink, M. F. B. G. No grip, no growth: the conceptual basis of excessive proteolysis in the treatment of cancer. Eur J Cancer, *36*: 1695-1705, 2000.

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Kranenburg, O., Kroon-Batenburg, L. M., Reijerkerk, A., Wu, Y. P., Voest, E. E., and Gebbink, M. F. Recombinant endostatin forms amyloid fibrils that bind and are cytotoxic to murine neuroblastoma cells in vitro. FEBS Lett, *539*: 149-155, 2003.

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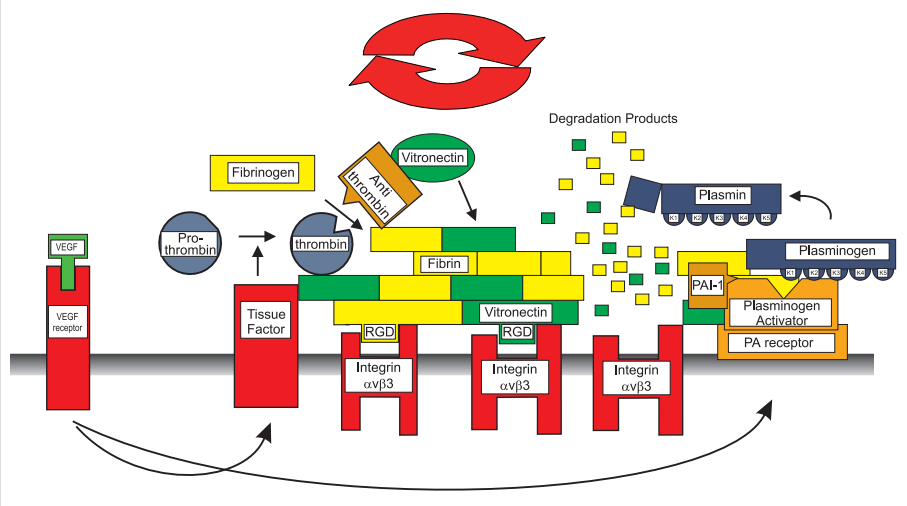
Reijerkerk, A., Meijers J. C.M., Havik, S. R., Bouma, B. N., Voest, E. E., Gebbink, M. F. B. G. Tumor growth and metastasis are not affected in Thrombin-activatable Fibrinolysis Inhibitor deficient mice. J Thromb Haemost. In press.

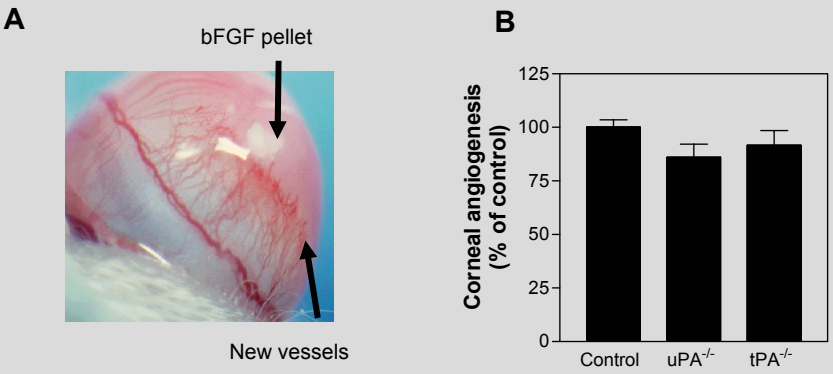
Color figures

Chapter 1, Figure 1.



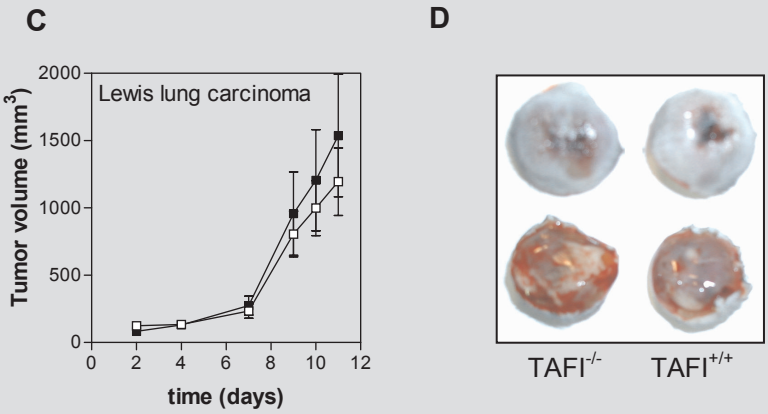
Coagulation and fibrinolysis on the cell surface. Upon stimulation by VEGF, components that initiate and control coagulation and fibrinolysis are upregulated. The continuous formation and breakdown of the provisional matrix is of great importance for cell viability, growth and motility. Matrix components 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).

Chapter 2, Figure 1.



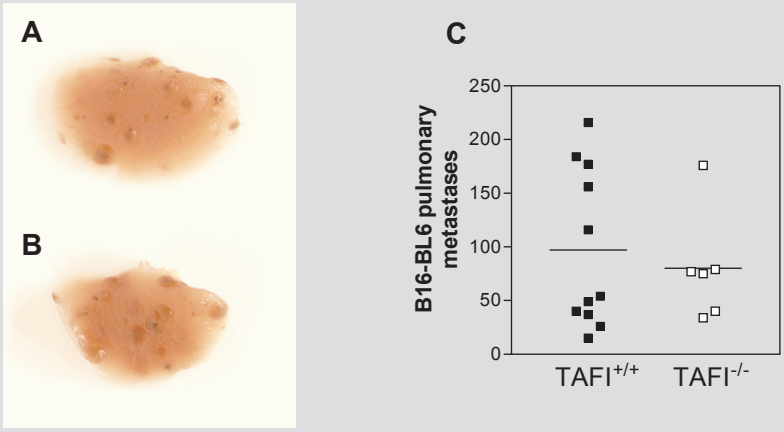
Corneal neovascularization in $uPA^{-/-}$, $tPA^{-/-}$ and control mice. (A) Photograph of a control mouse cornea 6 days after bFGF-coated pellet implantation. (B) bFGF-induced vessel formation in corneas of $uPA^{-/-}$ (n = 5), $tPA^{-/-}$ (n = 6) and control (n = 24) mice was analyzed 6 days after pellet implantation and quantified. Corneal angiogenesis was unaffected in the absence of tPA or uPA. Values are the normalized mean ± SEM.

Chapter 3, Figure 1.



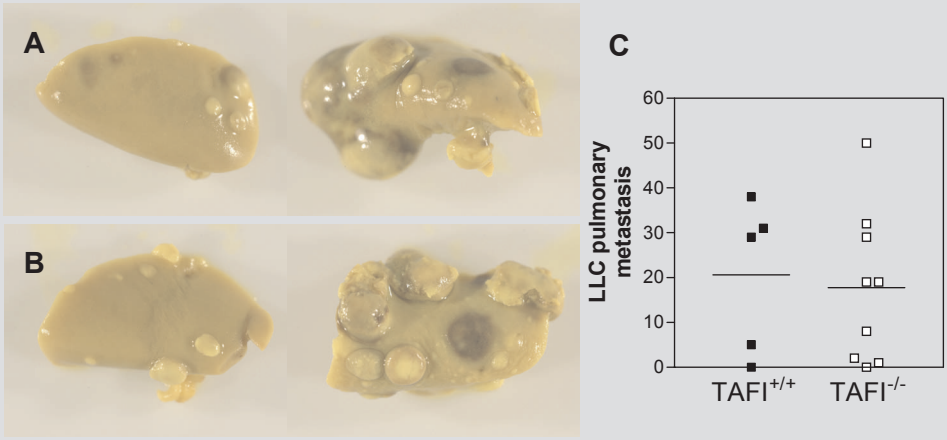
Effect of TAFI-deficiency on primary tumor growth. (C) 1 x 10^6 Lewis lung carcinoma cells were injected into the subcutaneous dorsa of mice and tumor volume was determined by calipation. No significant differences in tumor growth were observed between TAFI-deficient and wildtype mice. □ , $TAFI^{-/-}$; ■ , wildtype. (D) Representative example of subcutaneous LLC of a TAFI-deficient mouse (left panel) and a wildtype mouse (right panel) 11 days after transplantation. Upper panel: skin side facing up, Lower panel: basal side facing up.

Chapter 3, Figure 2 A-C.



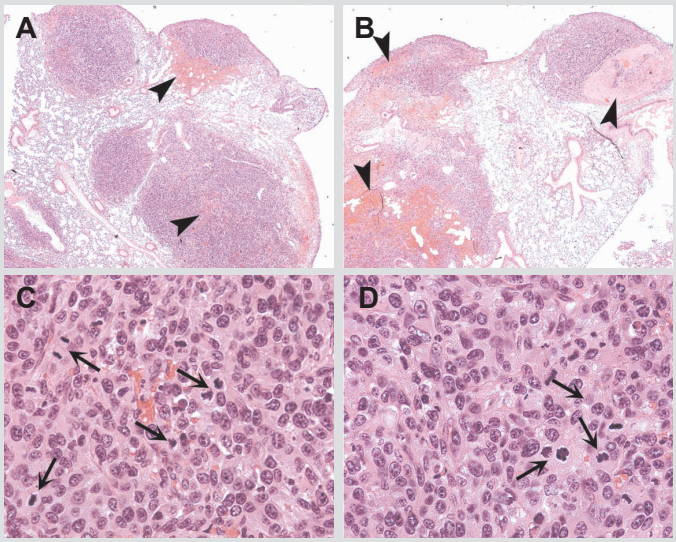
Effect of TAFI-deficiency on experimental pulmonary metastasis formation. 5 x 10^4 B16-BL6 melanoma cells were injected intravenously in TAFI-deficient and wildtype mice by tail vein injection. Lungs were isolated after 14 days, fixed and pulmonary metastasis formation was quantified by counting the number of tumor foci. (A,B) Representative examples of lungs with metastatic foci of B16-BL6 melanoma in wildtype (A) and TAFI-deficient mice (B) 14 days after intravenous injection. (C) The number of pulmonary nodules in TAFI-deficient and wildtype mice was not significantly different.

Chapter 3, Figure 3 A-C.



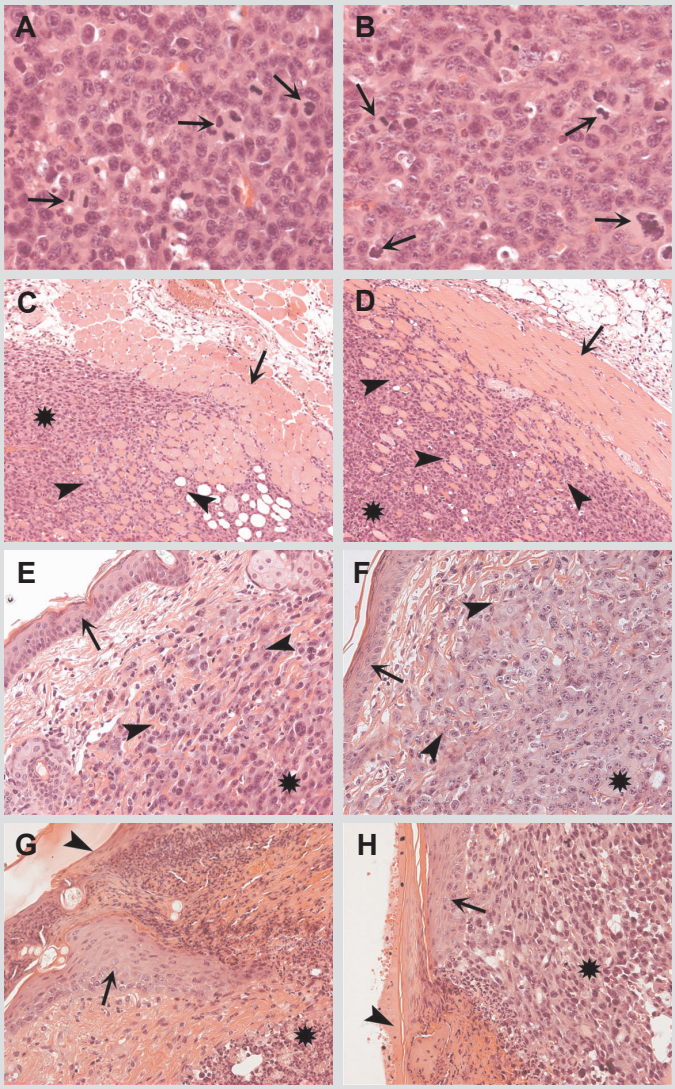
Effect of TAFI-deficiency on spontaneous pulmonary metastasis formation. TAFI-deficient and wildtype mice underwent primary tumor resection 11 days after subcutaneous transplantation of LLC. After 14 days the lungs were removed and fixed in Bouin's fixative. (A,B) Representative examples of metastatic pulmonary foci 14 days after tumor resection, indicating the presence of both small and large tumor nodules in wildtype (A) and TAFI-deficient (B) mice. (C) Quantification of the number of pulmonary metastatic foci revealed no significant difference between TAFI-deficient and wildtype mice.

Chapter 3, Figure 5.



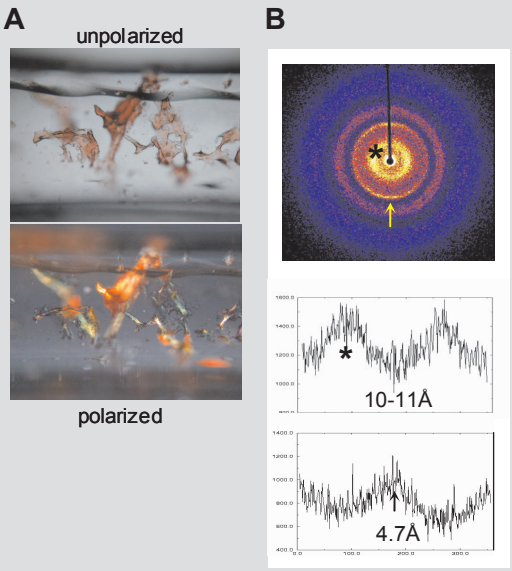
Histological analysis of lung metastases in TAFI-deficient (A,C) and wild-type (B,D) mice. Lungs were isolated 14 days after primary tumor resection, paraffin embedded, sectioned and stained with hematoxylin/eosin.
(A-D) Detailed analyses of the lungs revealed the presence of both parenchymal and pleural metastases composed of pleomorphic anaplastic cells having vesicular nuclei with multiple nucleoli and showing normal and abnormal mitotic figures (arrows) in TAFI-deficient and wildtype mice. Many tumor nodules had large necrotic areas associated with local hemorrhage (arrowheads).

Chapter 3, Figure 4.



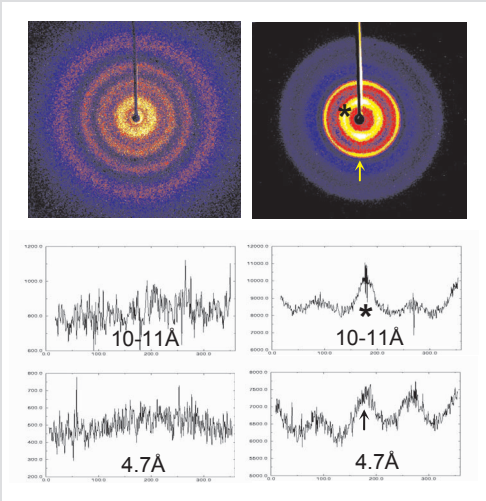
Histological analysis of primary tumors in TAFI-deficient (A,C,E,G) and wildtype (B,D,F,H) mice. Primary tumors of LLC were isolated 11 days after transplantation, paraffin embedded, sectioned and stained with hematoxylin/eosin. (A,B) Tumors in TAFI-deficient and wildtype mice both consist of closely packed anaplastic cells of different sizes with ample cytoplasm and show numerous normal as well as abnormal mitoses (arrows). (C,D) Tumors in both genotypes exhibited diffuse invasion into and destruction of adjacent muscle tissue. Arrows indicate normal muscle, arrowheads indicate tumor cell invasion surrounding individual myocytes. (E-F) Tumor invasion of the dermis. Arrows indicate normal epidermis. Arrowheads indicate infiltrating tumor cells in dermis. (G,H) Tumor invasion of the epidermis eventually leads to loss of keratinocytes and severe skin ulceration in both genotypes. Arrows indicate intact epidermis at the border of the ulceration. Arrowheads indicate crust formation. The asterisks indicate the tumor.

Chapter 6, Figure 1 A,B.



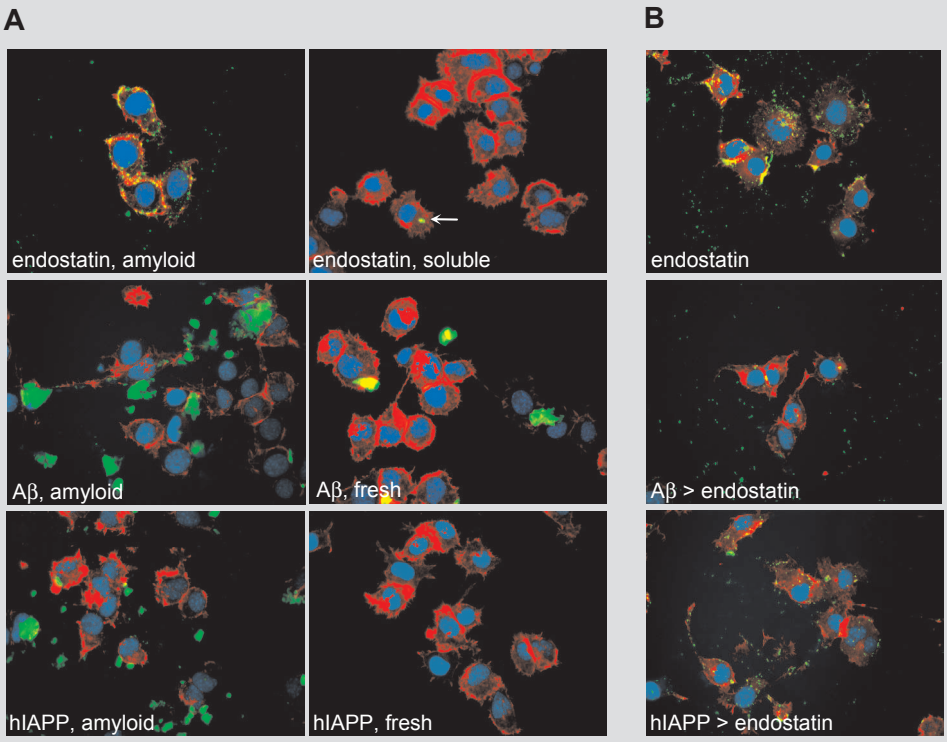
Amyloid properties of endostatin produced in bacteria. (A) Endostatin was purified from bacteria and stained with Congo Red. Samples were analyzed by light microscopy using both polarized and non-polarized light. The figure shows that endostatin binds Congo Red and exhibits green birefringence when examined under polarized light. (B) Endostatin samples were further analyzed by X-ray diffraction. The scattering as obtained on the 2D detector is color coded by intensity on a linear scale. The scattering intensity decreases as white-yellow-red-blue-black. The pattern shows diffraction maxima at 4.7 Å and 10-11 Å. The fiber axis (hydrogen bond direction) with 4.7 Å repeat is oriented vertically and is indicated by the arrow. The 10-11 Å repeat is preferentially oriented perpendicular to that as indicated by the asterisk. Tangential scans along the 2θ scattering angles corresponding to both d-spacings in the lower panel show that the scattering at 4.7 Å is oriented vertically and that at 10-11 Å horizontally.

Chapter 6, Figure 2.



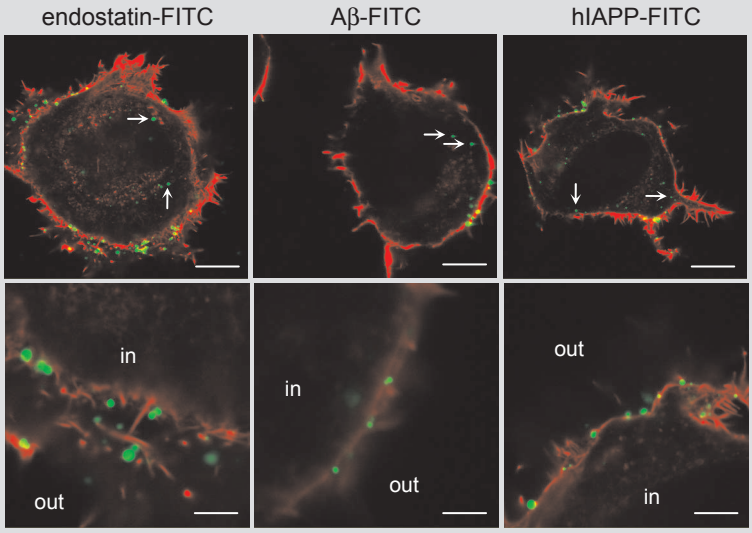
Conversion of soluble globular endostatin into amyloid endostatin. Endostatin pro-duced in yeast was dialysed against 8 M urea followed by extensive dialysis against H_2O. During dialysis endostatin pre-cipitates as a fine white solid. The samples were processed for X-ray diffraction analysis. (A) Diffraction pattern of untreated (yeast-produced) endostatin with no sign of cross-β sheet structure. (B) Diffraction pattern of urea-treated endostatin with extensive cross-β sheet formation. During solvent evaporation fibril formation occurred both vertically and horizontally in the capillary, as evidenced by the occurrence of peaks at 90, 180, 270 and 0/360 degrees in the tangential scans corresponding to both d-spacings. The asterisk indicates the peak reflection of the 10-11 Å d-spacing at 90^0. The arrow indicates the peak reflection of the 4.7 Å d spacing at 180^0.

Chapter 6, Figure 3.



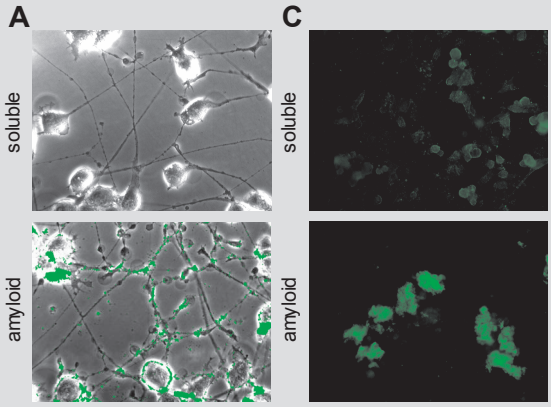
Binding of amyloid aggregates to N1E-115 neuroblastoma cells. (A) Neuronal N1E-115 cells, were incubated with either FITC-labeled aggregated amyloid endostatin, soluble endostatin, Aβ freshly resuspended, Aβ pre-aggregated for three weeks (Aβ amyloid), hIAPP freshly resuspended and hIAPP pre-aggregated for three weeks (hIAPP amyloid) (all at 5 μM) and washed. after 1 hour. The actin cytoskeleton was stained using Texas Red-conjugated palloidin (red) and the DNA with DAPI (blue). All amyloid aggregates (left panels) bound N1E-115 cells. (B) N1E-115 cells were pre-incubated for 6 hours with non-fluorescent Aβ or hIAPP (at 5 μM) and the binding of FITC-labelled amyloid endostatin (1 hour, 5 μM) was subsequently tested as above.

Chapter 6, Figure 4.



Localisation of cell-associated amyloid aggregates. N1E-115 cells were grown on glass coverslips and were incubated for 1 hour with FITC-labeled amyloid endostatin, Aβ and hIAPP. Actin was visualized using Texas Red-conjugated phalloidin. The coverslips were analyzed by confocal microscopy. Whole cell images (upper panel) show localisation of all three amyloids mainly to the cell surface. In addition, some of the aggregates show intracellular localization (arrows) (Bar, 10 μm). Zoomed images of cell surface areas (lower panel) shows that all three types of amyloid aggregates are in close proximity to the plasma membrane-bound cortical actin cytoskeleton (Bar, 2 μm).

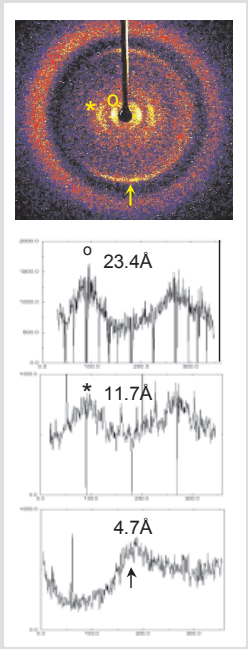
Chapter 6, Figure 5 A,C.



Toxicity of amyloid endostatin. (A) N1E-115 cells were differentiated into post-mitotic neurons on glass coverslips and were subsequently treated overnight with either FITC-labeled soluble or with FITC-labelled amyloid endostatin. Following extensive washing and fixation, binding of endostatin to the cells was visualized with fluorescence microscopy. Merged bright field and fluorescence images are shown. Only amyloid endostatin binds to the differentiated neuronal cells.

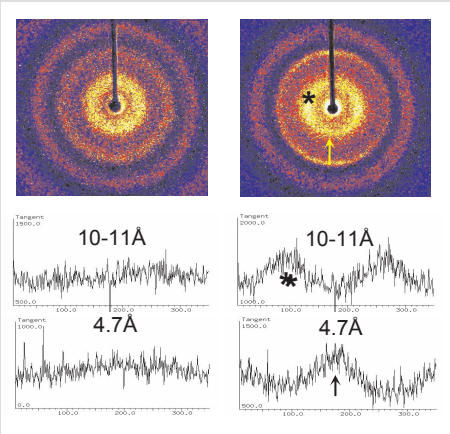
(C) N1E-115 cells were differentiated on glass coverslips and were subsequently treated with either soluble or with amyloid endostatin. Following fixation, the cells were stained with annexin V-FITC to visualize exposed phosphatidylserine. Detaching cell clusters that are associated with endostatin aggregates are highly positive for annexin V.

Chapter 7, Figure 2 D,B.



Structural analysis of fibrin-derived peptides. (B) Single drops of FP13 and FP10 (1 mg/ml in H_2O) were air-dried and stained with Congo Red according to the manufacturer's protocol. Microscopic analysis using non-polarized and polarized light shows that FP13, but not FP10 (not shown), binds Congo Red. (D) X-ray diffraction analysis of FP13. The scattering as obtained on the 2D detector is color-coded by intensity on a linear scale. The scattering intensity decreases as white-yellow-red-blue-black. The pattern shows diffraction maxima at 4.7, 11.7 and 23.4 Å. In the lower panels, tangential scans along the 2θ scattering angles corresponding to all three d-spacings show that the 4.7 Å scattering (hydrogen bonds) is oriented perpendicular to those at 11.7 and 23.4 Å (1x and 2x inter-sheet distance).

Chapter 7, Figure 4.



Enhanced plasminogen activation by cross-β–structured endostatin. (A) X-ray diffraction analysis of two distinct forms of endostatin. The left panel shows globular endostatin with no detectable cross-β sheets. The right panel shows insoluble endostatin with abundant cross-β sheets. The scattering as obtained on the 2D detector is color coded as in Figure 2D. The pattern shows diffraction maxima at 4.7 and 10-11 Å. The fiber axis (hydrogen bond direction) with 4.7 Å repeat is oriented vertically. The 10-11 Å repeat is preferentially oriented perpendicular to that, as indicated by the asterisk. Tangential scans along the 2θ scattering angles corresponding to both d-spacings in the lower panel show that the scattering at 4.7 Å is oriented vertically with the maximum intensity at 180^0 indicated by the arrow. The reflection at 10-11 Å is oriented horizontally with the maximum at 90^0 indicated by the asterisk.