

Melanotransferrin stimulates t-PA-dependent activation of plasminogen in endothelial cells leading to cell detachment

Yannève Rolland, Michel Demeule, Richard Béliveau *

Laboratoire de Médecine Moléculaire, Service d'Héματο-Oncologie, Hôpital Ste-Justine-UQAM, C.P. 8888, Succursale Centre-ville, Montréal, Québec, Canada H3C 3P8

Received 24 January 2006; received in revised form 20 March 2006; accepted 21 March 2006
Available online 19 April 2006

Abstract

Tissue plasminogen activator (t-PA) is an extracellular serine protease that converts the proenzyme plasminogen into the broad-spectrum substrate serine protease, plasmin. Plasmin, one of the most potent pro-angiogenic factors, is a key element in fibrinolysis, cell migration, tissue remodeling and tumor invasion. In the present investigation, we assessed the impact of the truncated form of soluble melanotransferrin (sMTf) on plasminogen activation by t-PA and subsequent endothelial cell detachment. Co-treatment of human endothelial microvessel cells with plasminogen, t-PA and sMTf significantly increased plasmin formation and activity in the culture medium. Plasmin generated in the presence of sMTf also led to a 30% reduction in fibronectin detection within cell lysates and to a 9-fold increase within the corresponding cell medium. Moreover, the presence of sMTf increases EC detachment by 6-fold compared to cells treated only with plasminogen and t-PA. Although the addition of α_2 -antiplasmin completely prevented plasmin formation and EC detachment, epigallocatechin gallate, GM6001 and a specific antibody directed against MMP-2 prevented cellular detachment without interfering with plasminogen activation. Overall, these data suggest that the anti-angiogenic properties of sMTf may result from local overstimulation of plasminogen activation by t-PA, thus leading to subsequent degradation of the Fn matrix and EC detachment. © 2006 Elsevier B.V. All rights reserved.

Keywords: Melanotransferrin; Plasminogen; Tissue-type plasminogen activator; Matrix metalloproteinase; Fibronectin; Endothelial cell; Detachment

1. Introduction

First identified as a major antigen in human melanomas [1,2], melanotransferrin (MTf) has been reported to be endogenously expressed by various cell types such as intestine and renal epithelial cells, salivary gland duct cells, liver endothelial cells (ECs), chondrocytes and cerebral endothelium [1,3]. To date, two variants of MTf have been identified: one that is secreted in a soluble form (sMTf) and the other that is associated with the cell membrane by a glycosylphosphatidyl inositol anchor (mMTf) [3,4]. Despite its significant homology to members of the transferrin family, MTf was shown to inefficiently transport iron into cells [2,5]. Recently, mMTf has also been reported to act as a key

player in cell surface plasminogen binding and in the activation processes involved during cell migration and invasion [6].

The angiogenic process leading to the formation of new capillaries from pre-existing blood vessels is an important step in embryonic development, tissue remodeling, wound healing, tumor growth and metastasis [7–13]. Most of these processes involve a cascade of serine proteinases and metalloproteinases (MMPs). Plasmin, one of the most potent pro-angiogenic factors, is a key element in fibrinolysis, cell migration, tissue remodeling and tumor invasion [14–16]. This major serine proteinase is generated following cleavage of the peptidic bond between Arg⁵⁶⁰ and Val⁵⁶¹ of plasminogen by urokinase (u-PA) or tissue-type (t-PA) plasminogen activators [17–20]. Traditionally, the role of t-PA has been considered to be confined to fibrinolysis whereas u-PA has been considered to be an activator of extracellular matrix (ECM) proteolysis [21,22]. However, other studies have suggested a role for t-

* Corresponding author.

E-mail address: oncomol@nobel.si.uqam.ca (R. Béliveau).

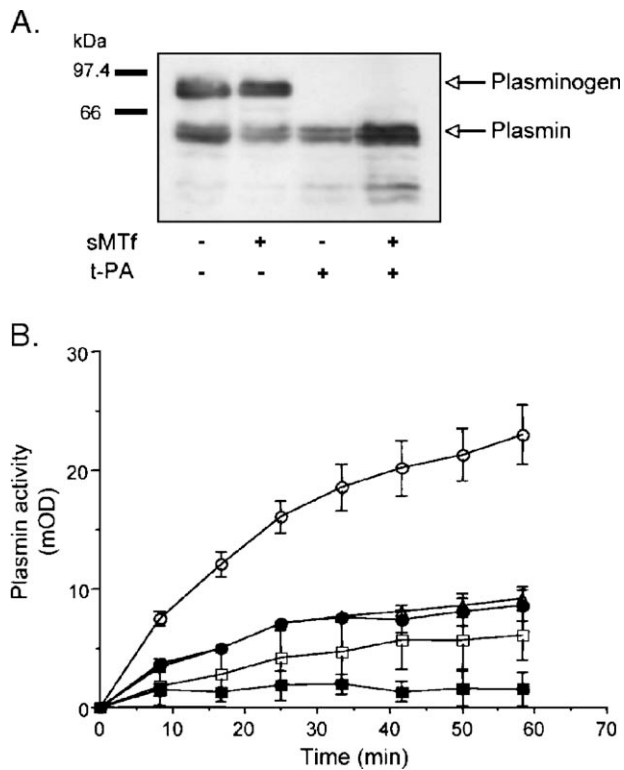


Fig. 1. sMTf treatment triggers plasmin formation in HMEC-1. (A) Immunodetection of plasmin in medium of cells treated with plasminogen and various combinations of sMTf and t-PA. (B) Plasmin activity was measured in conditioned medium of control cells (■) and cells treated with plasminogen (□), plasminogen combined with t-PA (●) or combined with t-PA and sMTf (○) supplemented with an IgG1 control (Δ), using a chromogenic substrate as described under Materials and methods. Values (mean \pm S.E.M.) represent $n = 3$.

PA in degradation of the ECM during angiogenesis [23]. Plasmin is involved in the control of cell proliferation [24], apoptosis [21,25] and migration [24] during the remodeling of vessel walls. In addition, plasmin can affect the ECM organization through the proteolysis of some components such as fibronectin [26], laminin [27,28], proteoglycans [29] as well as fibrin, and through the activation of MMPs [21].

MMPs are produced as latent pro-enzymes and require activation by limited proteolysis to perform their activities. MMPs can be associated with the cell membrane (MT-MMP) or secreted into the extracellular environment [30]. Two particular members of this family which are released by most epithelial and endothelial cells, gelatinases A and B (MMP-2 and MMP-9), are involved in the homeostasis of the ECM [31] and seem to play an important role in tumor invasion and metastasis [32]. Epithelial and endothelial cells require attachment to the ECM for cell survival [33]. Functional interactions have been described between plasminogen/plasmin and MMP systems, suggesting that both systems cooperate in tissue remodeling [34]. Moreover, MMPs such as pro-MMP-1 [29] and pro-MMP-3 [35] can be activated by plasmin [21,29].

The adhesive glycoprotein fibronectin (Fn) is a central component of the ECM involved in the regulation of adhesion-dependent survival signaling [36]. Fn binds to cell-surface matrix receptors through the Arg–Gly–Asp (RGD) cell binding site

[37,38]. Changes in the molecular structure and composition of the Fn matrix may provide new signals for regulating cell shape, migration and proliferation. Modulation of the Fn matrix may therefore be fundamental in the regulation of detachment-induced apoptosis, also called anoikis [37,39,40]. Although recent studies demonstrated little Fn degradation induced by plasmin, they established a strong link between plasmin activity and intact Fn in the material released from endothelial cells (EC) [41].

Several studies show a growing interest in recombinant human soluble MTf (sMTf), which is a C-terminal truncated form of the mMTf generated by the insertion of a stop codon after the Gly⁷¹¹ residue. These studies indicate that sMTf stimulates plasminogen activation by interacting with the single-chain zymogen pro-urokinase plasminogen activator (pro-uPA) and with plasminogen [42], leading to the inhibition of EC movement and tubulogenesis [43]. Considering these findings, we investigated the potential effect of sMTf on the activation of plasminogen in endothelial cells by the tissular-type plasminogen activator. Our results indicate that sMTf stimulates the formation of plasmin in the presence of t-PA and

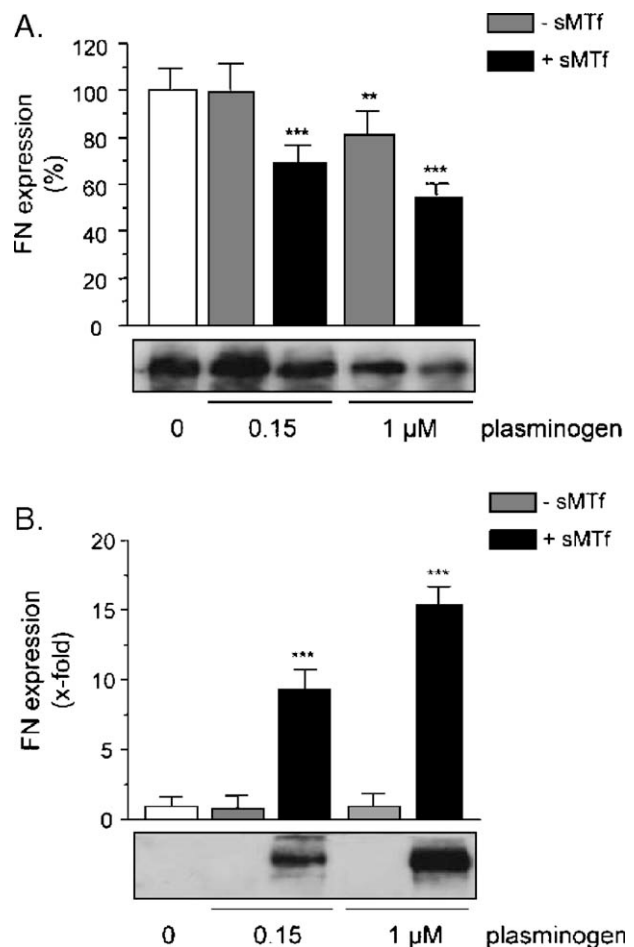


Fig. 2. Increase of plasmin formation by sMTf leads to the degradation of fibronectin. Fibronectin was detected by Western blot in HMEC-1 lysates (A) and concentrated media (B) from cells treated for 24 h with plasminogen and t-PA with (■) and without (□) sMTf as well as from control, untreated cells (□). Protein levels were quantified by densitometric analysis as described in Materials and methods. Values (mean \pm S.E.M.) are expressed relative to control cells; $n = 3$. ** $P < 0.005$ and *** $P < 0.0001$.

exogenous glu-plasminogen, the native circulating form of the zymogen, and affects the pericellular environment of endothelial cells by degrading the ECM and activating MMPs.

2. Materials and methods

2.1. Materials

Human recombinant soluble melanotransferrin (sMTf) was obtained from Biomarin Pharmaceutical (Novato, CA). Rabbit anti-human plasminogen IgG (364R) and glu-plasminogen (400) were purchased from American Diagnostica Inc. (Greenwich, CT). Human tissue plasminogen activator (t-PA) (612200) and GM6001 (364205) were from Calbiochem (La Jolla, CA). (–)-epigallocatechin gallate (EGCg) (E4143) and α_2 -antiplasmin (A8849) from human plasma were purchased from Sigma-Aldrich (Oakville, ON). Antibodies directed against human MMP-2 (MAB13405) and human fibronectin (F-3648) originated, respectively, from Chemicon International Inc. (Temecula, CA) and Sigma Immuno Chemicals (St. Louis, MO). The plasmin substrate used, VLK-pNA (S-2251), was purchased from Chromogenix (Milan, Italy).

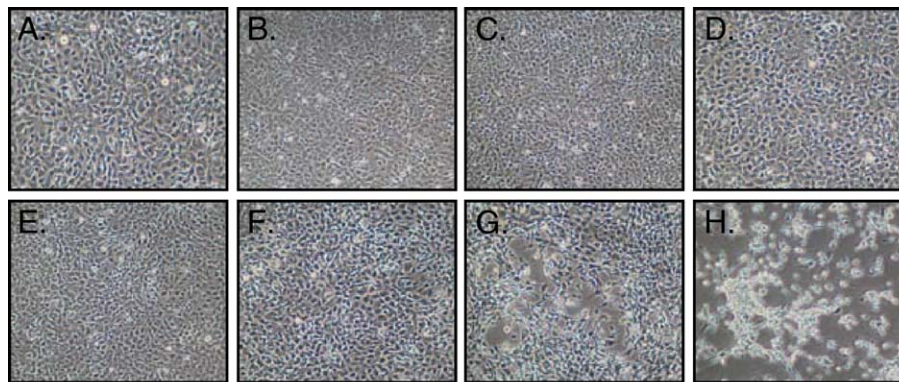
2.2. Cell culture

Human microvascular endothelial cells (HMEC-1) were obtained from the Center for Disease Control and Prevention (Atlanta, GA) and were cultured at 37 °C under 5% CO₂/95% air atmosphere in MCDB 131 medium (Sigma-

Aldrich) containing 10 mM L-glutamine, 10 ng/mL epidermal growth factor (EGF), 1 µg/mL hydrocortisone and 10% inactivated fetal bovine serum (FBS).

2.3. Endothelial cell treatment

HMEC-1 (5×10^5 cells) were seeded onto 6-well culture plates and grown to confluence in complete culture medium for 4 days. Cell treatment was performed for 24 h in the presence of fresh medium supplemented with (or lacking) tissue-type plasminogen activator (t-PA) (4 nM), glu-plasminogen (150 nM or 500 nM) and sMTf (100 nM). The inhibitory effects of α_2 -antiplasmin (150 nM), EGCg (1 µM) and GM6001 (10 µM) were also investigated. EC were also incubated with an antibody directed against human matrix metalloproteinase-2 (MMP-2) (20 µg/mL). Cells were then washed with a Ca²⁺-Mg²⁺-free phosphate saline buffer (PBS) and visualized by phase-contrast microscopy. Pictures were taken at a magnification of 100× using a digital Nikon Coolpix™ 5000 camera (Nikon Canada, Mississauga, ON) attached to a Nikon TMS-F microscope (Nikon Canada). Cells were then solubilized in lysis buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 1 mM ethylenediamine-tetraacetic acid (EDTA), 10 mM Tris, 2% N-octylglucoside, 1 mM orthovanadate, pH 7.5). Cell media were concentrated 10-fold using an Amicon Ultra-4 10,000 MWCO filter (Millipore Corporation, Bedford, MA). The viability of detached EC was determined by Trypan blue dye exclusion. Cellular detachment was analyzed by encircling the non-cell area in four different photographs using Adobe Photoshop version 8.0 (Adobe Systems Inc., CA) and quantified by calculating the percentage coverage of the selected area compared to non-treated cells using IPLab gel software, Scientific Image Processing 2.0a (Signal Analytics, VA).



I.

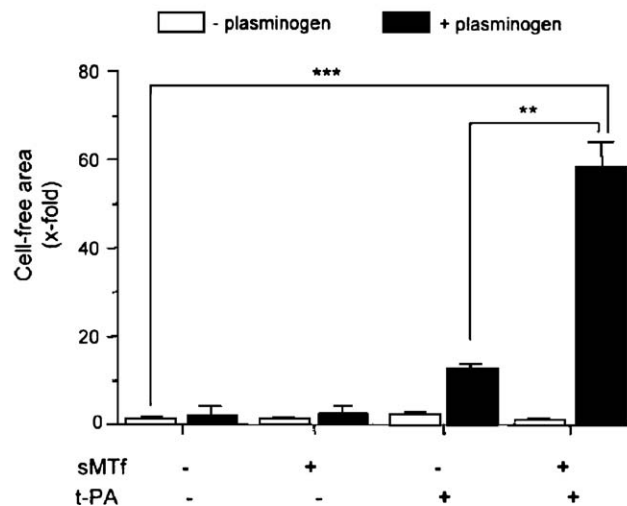


Fig. 3. Higher levels of plasmin induce endothelial cell detachment. Human microvascular endothelial cells (HMEC-1) (A) were incubated with sMTf (B), t-PA (C), sMTf and t-PA (D) and with plasminogen (E). Cell detachment was also assessed following co-treatment with various combinations of plasminogen with sMTf (F), t-PA (G) or both (H). After 24 h, cells were washed with PBS, photographed and analyzed. (I) Cell detachment was quantified in the absence (□) and presence (■) of plasminogen, as described under Materials and methods. Values (mean ± S.E.M.) are expressed relative to control cells; $n = 5$. ** $P < 0.005$ and *** $P < 0.0001$.

2.4. Plasmin activity

Plasmin activity was analyzed by adding 10 μ L of concentrated conditioned medium to the chromogenic plasmin substrate D-Val-Leu-Arg p-Nitroanilide (VLK-pNA) (15 μ g/assay) in reaction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 50 mM CaCl₂). The absorbance was monitored at 405 nm over 1 h at 37 °C with a Microplate Thermomax Autoreader (Molecular Devices, Sunnyvale, CA).

2.5. Western blot analysis

Conditioned media and cell lysates from HMEC-1 detachment were subjected to SDS-PAGE and separated proteins were transferred onto a polyvinylidene difluoride membrane (PVDF) (Perkin-Elmer Life Sciences, Boston, MA). Following transfer, immunodetection of plasminogen and fibronectin were performed. Proteins were quantified by laser densitometry using a ChemilmagerTM 5500 from Alpha Innotech Corporation (San Leandro, CA).

3. Results

3.1. sMTf stimulates plasmin formation

In light of the importance of sMTf and plasminogen interaction [6,42], we investigated the potential effects of sMTf on the EC plasminogen activation system. Media from cells treated with different combinations of plasminogen, t-PA and sMTf were subjected to Western blot analysis. The activation of plasminogen by t-PA results in the formation of plasmin, which migrates at approximately M_r 50,000 during gel electrophoresis (Fig. 1A). In the absence of treatment, ECs from human microvessels (HMEC-1) easily convert exogenous plasminogen into plasmin, as observed in the first lane of Fig. 1A. The addition of t-PA to the cell medium containing plasminogen leads to complete cleavage of the zymogen into plasmin. Moreover, the addition of sMTf to t-PA significantly increases the formation of plasmin in conditioned medium although the initial plasminogen concentration remained unchanged (Fig. 1A). As previously observed [43], t-PA seems to be a crucial player in this process since sMTf by itself, or combined with plasminogen, does not lead to the formation of plasmin (data not shown). Plasmin activity was also measured in concentrated media by using the conversion rate of VLK-pNA, a chromogenic substrate of plasmin (Fig. 1B). The results indicated that the plasmin observed by Western blot analysis possesses normal proteolytic activity. When sMTf is combined with plasminogen and t-PA, the VLK-pNA hydrolysis by EC is 3-fold higher than in conditioned medium containing only plasminogen and t-PA, and 4-fold higher than in conditioned medium with plasminogen alone. This difference between the levels of plasmin activity with or without sMTf supports the increased levels of plasmin detected in Fig. 1A. It is to note that the presence of a non-specific IgG control did not induce an increase of plasmin activity when added to plasminogen and t-PA, confirming that the effect of sMTf does not result in a protein effect.

3.2. Increased degradation of fibronectin by plasmin

We further investigated whether the higher levels of plasmin generated in the presence of sMTf would affect the structural components of the extracellular matrix (ECM). Since vitronec-

tin (Vn) and fibronectin (Fn) are two important components of the ECM involved in cell spreading and cell attachment [44], their degradation was studied in lysates of EC which had been treated with different concentrations of plasminogen. While vitronectin was not detected in quiescent nor treated HMEC-1 (data not shown), Western blot analysis showed a 30% reduction of Fn expression in the presence of t-PA, plasminogen and sMTf compared to incubation without sMTf (Fig. 2A). This reduction of Fn suggests that sMTf treatment induces the degradation of Fn by generated plasmin. Interestingly, higher concentrations of plasminogen led to a 45% reduction in Fn detection, while the same experiment performed with 500 nM of sMTf did not induce further changes in Fn expression over 24 h of treatment (data not shown). However, no degradation fragments could be detected by Western blot since the antibody used only recognizes the native Fn conformation. Furthermore, Western blot analysis of medium from HMEC-1 treated with t-PA and sMTf along with two different concentrations of plasminogen indicated 9- and 15-fold specific increases in native Fn (Fig. 2B).

3.3. Stimulation of EC detachment by plasmin

Previous studies indicated a strong link between plasmin formation and EC detachment [45,46]. Since our results show that sMTf stimulated the formation of plasmin in the presence of

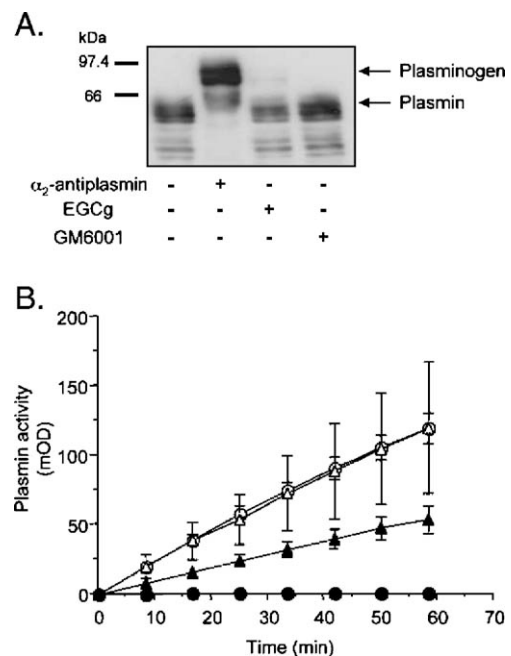


Fig. 4. Modulation of plasminogen activation by various inhibitors. (A) Plasmin formation was analyzed by Western blot in culture medium from HMEC-1 treated in the presence of plasminogen, sMTf and t-PA with or without α_2 -antiplasmin, EGCg or GM6001. (B) Plasmin activity was assayed from culture medium of cells co-treated with plasminogen, sMTf and tPA (○), to which α_2 -antiplasmin (●), EGCg (▲) or GM6001 (△) were added, using a chromogenic substrate as described in Materials and methods. Values (mean \pm S.E.M.) represent $n = 3$.

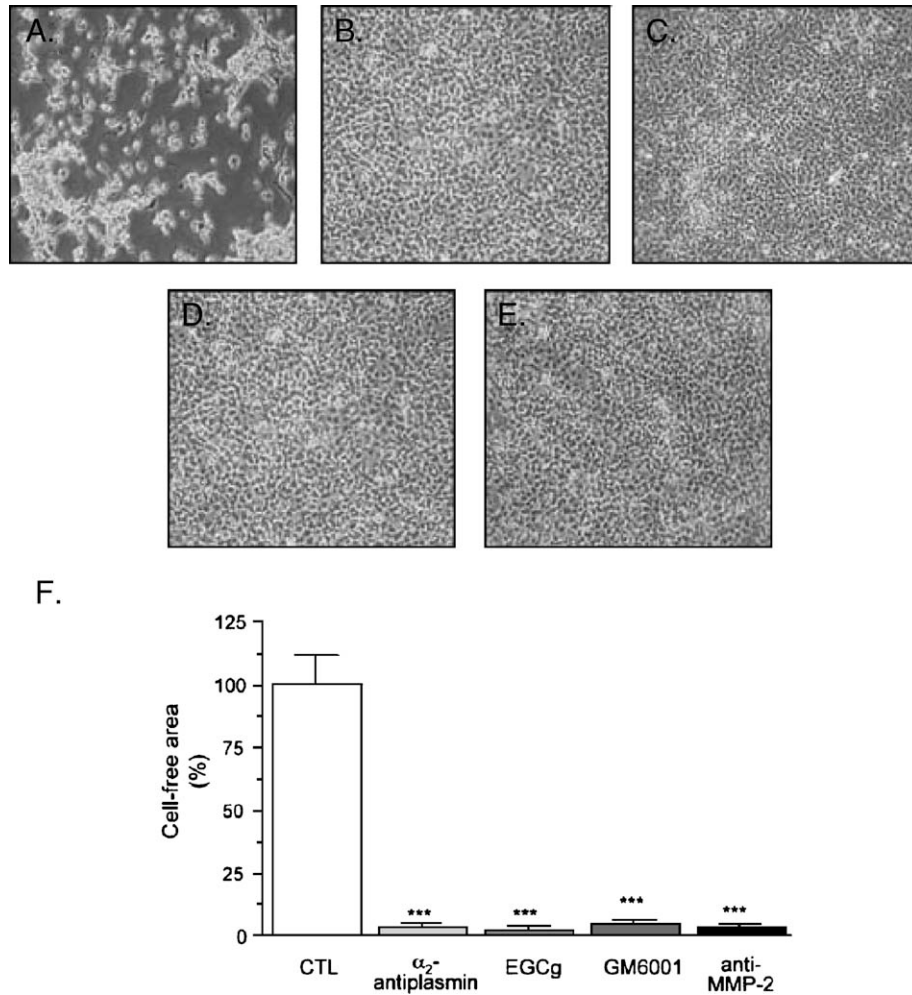


Fig. 5. The MMP activation system is a key player in EC detachment. Cells were incubated in the presence of plasminogen, sMTf and t-PA with or without α_2 -antiplasmin, EGCg or GM6001 (Ilomastat). HMEC-1 cells were also incubated in the presence of a specific antibody directed against human MMP-2 (control cells were incubated with a non-specific IgG1 antibody). After 24 h of treatment, cells were washed with PBS and photographed. Cell detachment was quantified as described in Materials and methods. Values (mean \pm S.E.M.) are expressed relative to sMTf, plasminogen and t-PA co-treated cells which are designated as 100%; $n = 5$. *** $P < 0.0001$.

plasminogen and t-PA, we studied whether the plasmin generated could lead to the detachment of HMEC-1. Cell adhesion was studied in the presence of various combinations of glu-plasminogen, t-PA and sMTf. While control cells (Fig. 3A) and cells treated with sMTf (Fig. 3B), t-PA (Fig. 3C) or plasminogen (Fig. 3E) did not show any morphological changes, co-treatment of EC with t-PA and plasminogen (Fig. 3G) induced an incomplete rounding up of the cells after 24 h. Furthermore, the addition of sMTf (Fig. 3H) generated dramatic changes in cell shape leading to subsequent cellular detachment. Interestingly, co-treatment of HMEC-1 with sMTf and t-PA (Fig. 3D) or with sMTf and plasminogen (Fig. 3F) did not alter the adhesive properties of HMEC-1 compared to untreated cells. The EC detachment observed eventually led to cellular death, since no living cells were observed, using trypan blue dye exclusion, among the detached HMEC-1 (data not shown). Quantification of EC detachment in Fig. 3I shows that the addition of sMTf to HMEC-1 exposed to plasminogen and t-PA increased cellular detachment from 10- to 60-fold compared to untreated cells.

3.4. Modulation of plasmin formation

In order to demonstrate that plasmin is not the only factor responsible for EC detachment, HMEC-1 cells were treated with various protease inhibitors known to act on different proteinase systems. α_2 -antiplasmin was demonstrated to prevent plasmin formation and to inhibit the activity of plasmin formed [47,48]. Western blot analysis shows that the addition of α_2 -antiplasmin completely impeded the formation of plasmin in conditioned medium, while neither GM6001 nor epigallocatechin gallate (EGCg) were able to completely prevent plasmin formation (Fig. 4A), although EGCg significantly decreased plasmin formation with a slight signal attributed to plasminogen being detected by Western blotting (Fig. 4A). It has been reported that EGCg inhibits both plasmin and MMP activities whereas GM6001, a broad range MMP inhibitor, does not interfere with plasmin formation. In agreement with Western blot analysis, treatment of ECs with EGCg and α_2 -antiplasmin decreased plasmin activity by 50% and 100%, respectively, compared to cells exposed to

t-PA, plasminogen and sMTf (Fig. 4B). Treatment of HMEC-1 with GM6001 did not induce any modulation in the activity of plasmin, which further confirms the specificity of this inhibitor for the MMP activation system.

3.5. Effects of various protease inhibitors on EC detachment

Since EC detachment mediated by sMTf is mainly due to the action of plasmin, which is also known to stimulate the activation of pro-MMPs, the role of MMPs in this process was further examined. The addition of α 2-antiplasmin and EGCg completely prevented cellular detachment of HMEC-1 while the presence of GM6001 decreased cell detachment by 95% (Fig. 5). These results suggest that MMPs are also involved in EC detachment induced by sMTf.

While several cultured endothelial cells strongly express MMP-1,-2,-3,-9 and-14 [49], HMEC-1 express high levels of MMP-2, while no MMP-9 activity could be detected by zymography (data not shown). An antibody directed against the human form of MMP-2 was used to assess the potential role of this gelatinase in EC detachment. The addition of anti-MMP-2 prevented EC detachment by 93% compared to cells treated with a non-specific IgG₁ antibody (Fig. 5).

4. Discussion

The human MTF shows strong homology with the iron transporter named transferrin. Although these proteins possess similar structure, they were demonstrated to own distinct role, notably for iron transport. MTF is known to be either associated to the cell membrane or secreted in the extracellular environment. Previous studies reported that transferrin and MTF could stimulate angiogenesis *in vitro* and *in vivo* [50,51]. However, in these studies, soluble transferrin and recombinant MTF were used either as a chemoattractant during chemotaxis and chemoinvasion assays, or adsorbed on gelatine sponges in chick embryo chorio-allantoic membrane assays. The activity of a protein may depend on numerous factors, such as three-dimensional structure. These studies indicate that a MTF gelatine-adsorbed protein will not exert the same properties than the equivalent soluble form. Moreover, a gelatin-adsorbed protein could be considered as non-physiologic conditions. Human and bovine lactoferrin were shown to inhibit tumor-induced angiogenesis [52] and to suppress tumor growth and metastasis in the mouse and rat [53–56]. Recent work from our team demonstrated that the binding of plasminogen to membrane-bound melanotransferrin could favour its conversion into plasmin by plasminogen activator, such as u-PA, located in the vicinity of the cell surface, thereby promoting cell migration and invasion [6]. Our previous results reported that sMTf treatment reduces the regeneration of free and active urokinase plasminogen activator receptor (u-PAR) at the cell surface, by both increasing the internalization of his scavenger receptor the low-density lipoprotein-related protein (LRP) and reducing its protein expression [43]. Considering the importance of plasminogen activation at the cell surface, the modulation of plasmin generation may take an important place in various processes leading to angiogenesis.

Activation of plasminogen, leading to plasmin formation, is associated with extracellular matrix (ECM) degradation as it occurs in blood clot dissolution, tissue remodeling, invasive growth of cancer cells and angiogenesis [57–60]. Plasmin also mediates proteolysis indirectly by the activation of MMPs, which further degrade the ECM. Induction of plasminogen activation leads to EC detachment [45], inhibition of cell adhesion [20] and overstimulation of this system may result in excessive matrix degradation and EC death [61].

Among plasminogen activators, we already demonstrated that sMTf could affect molecular processes involving u-PA-dependent plasminogen activation [43]. Here we demonstrate that sMTf can also stimulate plasminogen activation by t-PA. Our results suggest that sMTf could protect plasmin against internalization and/or degradation, notably at the kringle domain level. Regulation of both the plasmin activity and the conversion of plasminogen into plasmin are critical to avoid inappropriate tissue damage and proteolysis of the ECM. Various mechanisms are known to be involved in the regulation of plasmin activity. The physiological inhibitor of plasmin, α 2-antiplasmin, rapidly inactivates fluid-phase plasmin by forming a tight complex with the proteinase [62–64]. Activated MMPs can also digest and inactivate plasmin [65]. In addition, plasmin is capable of autoprolysis [66], which can be retarded by fibrinogen and ϵ -aminocaproic acid [67,68]. Although recent work from our laboratory has established that plasminogen strongly interacts with immobilized sMTf [42], further studies are needed to clarify the molecular mechanism by which sMTf could facilitate plasminogen activation, delay plasmin degradation and allow further activation of plasminogen and pro-MMPs.

Under pathological conditions, plasmin could be generated *in situ* at the cell surface and be responsible for inducing anoikis by degrading pericellular adhesive glycoproteins such as fibronectin, vitronectin or laminin, which participate in cell anchorage and survival signaling. Anoikis is a form of apoptosis induced by detachment of adherent cells from the ECM [69]. Fibronectin is known to regulate adhesion-dependent survival signaling [36] and to play an important role in several cellular processes including adhesion, migration, differentiation and growth [44]. A recent study reported that high levels of plasmin caused a significant decrease of intact Fn in EC, but also a concomitant increase of intact Fn in the cell medium [41]. The reduction of Fn detection in HMEC-1 cells treated with plasminogen, t-PA and sMTf in the present study may result from significant cleavage by plasmin since detection of intact Fn is increased in the corresponding cell media. Higher levels of plasmin generated in the presence of sMTf could greatly affect the ECM components and thus decrease cell anchorage to the ECM. Furthermore, modulation of the Fn matrix is fundamental in the regulation of detachment-induced apoptosis [37,39,40]. Similar results have been reported, where amyloid endostatin, a co-factor for t-PA-mediated plasminogen activation, induced endothelial cell-mediated plasmin formation resulting in vitronectin degradation, cell remodeling and detachment [70,71]. Therefore, we suggest that sMTf acts through a similar mechanism on the Fn matrix.

We further investigated other players downstream to plasmin that may be implicated in the molecular events triggered by sMTf. Members of the MMP family are crucial in ECM and basement membrane degradation during angiogenesis. It has already been reported that EC detachment could be plasmin-dependent, without stimulating the MMP activation system [70]. Therefore, the authors stipulated that the plasmin generated might not be sufficient to induce the activation of pro-MMPs. In contrast, our results show that sMTf stimulates plasminogen activation by t-PA in a manner sufficient to induce the activation of pro-MMPs, considering that EGCg and GM6001 are both capable of preventing HMEC-1 detachment.

Since MMPs seem to play an important role in the EC detachment induced by sMTf, we sought to identify the involvement of a specific proteinase. Our data from experiments done on quiescent HMEC-1 cells show that these cells constitutively and predominantly express MMP-2. Although the role of plasmin in the activation of pro-MMP-2 remains unclear, several reports have demonstrated that plasmin can activate pro-MMP-2 in the presence of MT1-MMP [72–75]. Furthermore, the activation of MMP-2 was reported to be stimulated in human umbilical EC (HUVEC) during apoptosis [76]. Since treatment of HMEC-1 with a specific antibody directed against MMP-2 prevented cellular detachment, we suggest that MMP-2 activity strongly contributes to local digestion of the ECM and, eventually, of the Fn matrix. Consequently, the presence of sMTf in cell culture medium conducts to more efficient formation of plasmin and stimulates the activity of MMP-2, leading to increased Fn matrix degradation and finally subsequent endothelial cell death following major cellular remodeling and detachment from the ECM.

Although the activity of t-PA was conventionally associated with fibrinolysis [21], recent work suggested a role in ECM degradation during angiogenesis [23]. Furthermore, numerous studies have revealed high levels of t-PA expression in several human tumors [77–81], associated with good prognosis in cancer patients [82–84]. We report here that overstimulation of the plasminogen activation system by sMTf leads to major detachment of endothelial cells. Since t-PA expression by EC is induced by angiogenic factors, including basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) [85], EC detachment and subsequent cell death should play an important role in the angiogenic process. In fact, therapeutic administration of sMTf may result in the stimulation of plasminogen activation by t-PA produced in the tumor and interfere in EC proliferation and survival.

Taken together, overstimulation of the plasminogen activation system locally by agents such as sMTf may result in excessive matrix degradation and endothelial cell death, thereby preventing angiogenesis and tumor growth [71]. Although we have previously demonstrated that sMTf reduces HMEC-1 capacity to generate plasmin from plasminogen by disturbing the u-PAR/LRP plasminolytic system, the results shown here indicate that, under specific conditions, such as an angiogenic environment, sMTf may lead to EC detachment. These findings constitute a novel pathway for interference in tumor growth and show interesting therapeutic applications for human recombinant melanotransferrin.

Acknowledgements

We thank Dr. Anthony Régina and Dr. Denis Gingras for their critical reading of this manuscript. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to Richard Béliveau.

References

- [1] J.P. Brown, R.G. Woodbury, C.E. Hart, I. Hellstrom, K.E. Hellstrom, Quantitative analysis of melanoma-associated antigen p97 in normal and neoplastic tissues, *Proc. Natl. Acad. Sci. U. S. A.* 78 (1981) 539–543.
- [2] J.P. Brown, R.M. Hewick, I. Hellstrom, et al., Human melanoma-associated antigen p97 is structurally and functionally related to transferrin, *Nature* 296 (1982) 171–173.
- [3] R. Alemany, M.R. Vila, C. Franci, et al., Glycosyl phosphatidylinositol membrane anchoring of melanotransferrin (p97): apical compartmentalization in intestinal epithelial cells, *J. Cell Sci.* 104 (Pt. 4) (1993) 1155–1162.
- [4] M.R. Food, S. Rothenberger, R. Gabathuler, et al., Transport and expression in human melanomas of a transferrin-like glycosylphosphatidylinositol-anchored protein, *J. Biol. Chem.* 269 (1994) 3034–3040.
- [5] D.R. Richardson, The role of the membrane-bound tumour antigen, melanotransferrin (p97), in iron uptake by the human malignant melanoma cell, *Eur. J. Biochem.* 267 (2000) 1290–1298.
- [6] J. Michaud-Levesque, M. Demeule, R. Béliveau, Stimulation of cell surface plasminogen activation by membrane-bound melanotransferrin: a key phenomenon for cell invasion, *Exp. Cell Res.* 308 (2) (2005) 479–490.
- [7] L.F. Brown, M. Detmar, K. Claffey, et al., Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine, *EXS* 79 (1997) 233–269.
- [8] P. Carmeliet, Angiogenesis in health and disease, *Nat. Med.* 9 (2003) 653–660.
- [9] H.F. Dvorak, Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy, *J. Clin. Oncol.* 20 (2002) 4368–4380.
- [10] H.F. Dvorak, Rous-Whipple Award Lecture. How tumors make bad blood vessels and stroma, *Am. J. Pathol.* 162 (2003) 1747–1757.
- [11] H.F. Dvorak, J.A. Nagy, D. Feng, L.F. Brown, A.M. Dvorak, Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis, *Curr. Top. Microbiol. Immunol.* 237 (1999) 97–132.
- [12] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, *Nat. Med.* 9 (2003) 669–676.
- [13] J. Folkman, Role of angiogenesis in tumor growth and metastasis, *Semin. Oncol.* 29 (2002) 15–18.
- [14] F.J. Castellino, V.A. Ploplis, Structure and function of the plasminogen/plasmin system, *Thromb. Haemostasis* 93 (2005) 647–654.
- [15] P.A. Andreasen, L.S. Nielsen, J. Grondahl-Hansen, et al., Inactive proenzyme to tissue-type plasminogen activator from human melanoma cells, identified after affinity purification with a monoclonal antibody, *EMBO J.* 3 (1984) 51–56.
- [16] G. Markus, The relevance of plasminogen activators to neoplastic growth, A review of recent literature, *Enzyme* 40 (1988) 158–172.
- [17] T.L. Moser, M.S. Stack, M.L. Wahl, S.V. Pizzo, The mechanism of action of angiotensin: can you teach an old dog new tricks? *Thromb. Haemostasis* 87 (2002) 394–401.
- [18] Y.V. Parfyonova, O.S. Plekhanova, V.A. Tkachuk, Plasminogen activators in vascular remodeling and angiogenesis, *Biochemistry* 67 (2002) 119–134 (Mosc).
- [19] J.D. Vassalli, M.S. Pepper, Tumour biology, Membrane proteases in focus, *Nature* 370 (1994) 14–15.
- [20] J. Reinartz, B. Schafer, R. Batrla, C.E. Klein, M.D. Kramer, Plasmin abrogates alpha v beta 5-mediated adhesion of a human keratinocyte cell line (HaCaT) to vitronectin, *Exp. Cell Res.* 220 (1995) 274–282.

- [21] P. Carmeliet, L. Moons, R. Lijnen, et al., Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation, *Nat. Genet.* 17 (1997) 439–444.
- [22] P. Carmeliet, D. Collen, Development and disease in proteinase-deficient mice: role of the plasminogen, matrix metalloproteinase and coagulation system, *Thromb. Res.* 91 (1998) 255–285.
- [23] Y. Sato, K. Okamura, A. Morimoto, et al., Indispensable role of tissue-type plasminogen activator in growth factor-dependent tube formation of human microvascular endothelial cells in vitro, *Exp. Cell Res.* 204 (1993) 223–229.
- [24] J.M. Herbert, I. Lamarche, P. Carmeliet, Urokinase and tissue-type plasminogen activator are required for the mitogenic and chemotactic effects of bovine fibroblast growth factor and platelet-derived growth factor-BB for vascular smooth muscle cells, *J. Biol. Chem.* 272 (1997) 23585–23591.
- [25] J.M. Herbert, P. Carmeliet, Involvement of u-PA in the anti-apoptotic activity of TGFbeta for vascular smooth muscle cells, *FEBS Lett.* 413 (1997) 401–404.
- [26] L.A. Liotta, R.H. Goldfarb, R. Brundage, et al., Effect of plasminogen activator (urokinase), plasmin, and thrombin on glycoprotein and collagenous components of basement membrane, *Cancer Res.* 41 (1981) 4629–4636.
- [27] O. Saksela, Plasminogen activation and regulation of pericellular proteolysis, *Biochim. Biophys. Acta* 823 (1985) 35–65.
- [28] C.S. He, S.M. Wilhelm, A.P. Pentland, et al., Tissue cooperation in a proteolytic cascade activating human interstitial collagenase, *Proc. Natl. Acad. Sci. U. S. A.* 86 (1989) 2632–2636.
- [29] E. Mochan, T. Keler, Plasmin degradation of cartilage proteoglycan, *Biochim. Biophys. Acta* 800 (1984) 312–315.
- [30] M.S. Pepper, Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis, *Arterioscler. Thromb. Vasc. Biol.* 21 (2001) 1104–1117.
- [31] M.A. Forget, R.R. Desrosiers, R. Beliveau, Physiological roles of matrix metalloproteinases: implications for tumor growth and metastasis, *Can. J. Physiol. Pharmacol.* 77 (1999) 465–480.
- [32] D.E. Kleiner, W.G. Stetler-Stevenson, Matrix metalloproteinases and metastasis, *Cancer Chemother. Pharmacol.* 43 (1999) S42–S51 (Suppl.).
- [33] S.M. Frisch, H. Francis, Disruption of epithelial cell–matrix interactions induces apoptosis, *J. Cell Biol.* 124 (1994) 619–626.
- [34] X. Houard, C. Monnot, V. Dive, P. Corvol, M. Pagano, Vascular smooth muscle cells efficiently activate a new proteinase cascade involving plasminogen and fibronectin, *J. Cell. Biochem.* 88 (2003) 1188–1201.
- [35] H. Nagase, J.J. Enghild, K. Suzuki, G. Salvesen, Stepwise activation mechanisms of the precursor of matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl)mercuric acetate, *Biochemistry* 29 (1990) 5783–5789.
- [36] J.L. Sechler, J.E. Schwarzbauer, Control of cell cycle progression by fibronectin matrix architecture, *J. Biol. Chem.* 273 (1998) 25533–25536.
- [37] H.L. Hadden, C.A. Henke, Induction of lung fibroblast apoptosis by soluble fibronectin peptides, *Am. J. Respir. Crit. Care Med.* 162 (2000) 1553–1560.
- [38] C.D. Buckley, D. Pilling, N.V. Henriquez, et al., RGD peptides induce apoptosis by direct caspase-3 activation, *Nature* 397 (1999) 534–539.
- [39] E.A. Verderio, D. Telci, A. Okoye, G. Melino, M. Griffin, A novel RGD-independent cell adhesion pathway mediated by fibronectin-bound tissue transglutaminase rescues cells from anoikis, *J. Biol. Chem.* 278 (2003) 42604–42614.
- [40] J. Jeong, I. Han, Y. Lim, et al., Rat embryo fibroblasts require both the cell-binding and the heparin-binding domains of fibronectin for survival, *Biochem. J.* 356 (2001) 531–537.
- [41] A. Bonnefoy, C. Legrand, Proteolysis of subendothelial adhesive glycoproteins (fibronectin, thrombospondin, and von Willebrand factor) by plasmin, leukocyte cathepsin G, and elastase, *Thromb. Res.* 98 (2000) 323–332.
- [42] M. Demeule, Y. Bertrand, J. Michaud-Levesque, et al., Regulation of plasminogen activation: a role for melanotransferrin (p97) in cell migration, *Blood* 102 (2003) 1723–1731.
- [43] J. Michaud-Levesque, Y. Rolland, M. Demeule, Y. Bertrand, R. Beliveau, Inhibition of endothelial cell movement and tubulogenesis by human recombinant soluble melanotransferrin: involvement of the u-PAR/LRP plasminolytic system, *Biochim. Biophys. Acta* 1743 (2005) 243–253.
- [44] J.L. Guan, R.O. Hynes, Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor alpha 4 beta 1, *Cell* 60 (1990) 53–61.
- [45] M. Ge, G. Tang, T.J. Ryan, A.B. Malik, Fibrinogen degradation product fragment D induces endothelial cell detachment by activation of cell-mediated fibrinolysis, *J. Clin. Invest.* 90 (1992) 2508–2516.
- [46] G.E. Davis, K.A. Pintar-Allen, R. Salazar, S.A. Maxwell, Matrix metalloproteinase-1 and-9 activation by plasmin regulates a novel endothelial cell-mediated mechanism of collagen gel contraction and capillary tube regression in three-dimensional collagen matrices, *J. Cell Sci.* 114 (2001) 917–930.
- [47] M. Levi, D. Roem, A.M. Kamp, et al., Assessment of the relative contribution of different protease inhibitors to the inhibition of plasmin in vivo, *Thromb. Haemostasis* 69 (1993) 141–146.
- [48] T. Syrovets, T. Simmet, Novel aspects and new roles for the serine protease plasmin, *Cell Mol. Life Sci.* 61 (2004) 873–885.
- [49] M.A. Moses, The regulation of neovascularization of matrix metalloproteinases and their inhibitors, *Stem Cells* 15 (1997) 180–189.
- [50] M.F. Carlevaro, A. Albini, D. Ribatti, et al., Transferrin promotes endothelial cell migration and invasion: implication in cartilage neovascularization, *J. Cell Biol.* 136 (1997) 1375–1384.
- [51] R. Sala, W.A. Jefferies, B. Walker, et al., The human melanoma associated protein melanotransferrin promotes endothelial cell migration and angiogenesis in vivo, *Eur. J. Cell Biol.* 81 (2002) 599–607.
- [52] M. Shimamura, Y. Yamamoto, H. Ashino, et al., Bovine lactoferrin inhibits tumor-induced angiogenesis, *Int. J. Cancer* 111 (2004) 111–116.
- [53] J. Bezault, R. Bhimani, J. Wiprovnick, P. Furmanski, Human lactoferrin inhibits growth of solid tumors and development of experimental metastases in mice, *Cancer Res.* 54 (1994) 2310–2312.
- [54] Y.C. Yoo, S. Watanabe, R. Watanabe, et al., Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice, *Jpn. J. Cancer Res.* 88 (1997) 184–190.
- [55] M. Iigo, T. Kuhara, Y. Ushida, et al., Inhibitory effects of bovine lactoferrin on colon carcinoma 26 lung metastasis in mice, *Clin. Exp. Metastasis* 17 (1999) 35–40.
- [56] K. Norrby, I. Mattsby-Baltzer, M. Innocenti, S. Tuneberg, Orally administered bovine lactoferrin systemically inhibits VEGF(165)-mediated angiogenesis in the rat, *Int. J. Cancer* 91 (2001) 236–240.
- [57] M.J. Duffy, Plasminogen activators and cancer, *Blood Coagul. Fibrinolysis* 1 (1990) 681–687.
- [58] M.D. Kramer, J. Reinartz, G. Brunner, V. Schirmacher, Plasmin in pericellular proteolysis and cellular invasion, *Invasion Metastasis* 14 (1994) 210–222.
- [59] G. Pintucci, A. Bikfalvi, S. Klein, D.B. Rifkin, Angiogenesis and the fibrinolytic system, *Semin. Thromb. Hemostasis* 22 (1996) 517–524.
- [60] W.R. Bell, The fibrinolytic system in neoplasia, *Semin. Thromb. Hemostasis* 22 (1996) 459–478.
- [61] M. Sugimura, H. Kobayashi, T. Terao, Plasmin modulators, aprotinin and anti-catalytic plasmin antibody, efficiently inhibit destruction of bovine vascular endothelial cells by choriocarcinoma cells, *Gynecol. Oncol.* 52 (1994) 337–346.
- [62] I. Rakoczi, B. Wiman, D. Collen, On the biological significance of the specific interaction between fibrin, plasminogen and antiplasmin, *Biochim. Biophys. Acta* 540 (1978) 295–300.
- [63] D. Rouy, E. Angles-Cano, The mechanism of activation of plasminogen at the fibrin surface by tissue-type plasminogen activator in a plasma milieu in vitro, Role of alpha 2-antiplasmin, *Biochem. J.* 271 (1990) 51–57.
- [64] C. Longstaff, P.J. Gaffney, Serpin–serine protease binding kinetics: alpha 2-antiplasmin as a model inhibitor, *Biochemistry* 30 (1991) 979–986.
- [65] R. Mazzieri, L. Masiero, L. Zanetta, et al., Control of type IV collagenase activity by components of the urokinase–plasmin system: a regulatory mechanism with cell-bound reactants, *EMBO J.* 16 (1997) 2319–2332.
- [66] J. Jespersen, J. Gram, T. Astrup, The autodigestion of human plasmin follows a bimolecular mode of reaction subject to product inhibition, *Thromb. Res.* 41 (1986) 395–404.
- [67] P.J. Gaffney, Fibrin(-ogen) interactions with plasmin, *Haemostasis* 6 (1977) 2–25.
- [68] M. Grimard, Evidence for high-molecular weight active peptides originating from porcine plasmin autolysis, *Biochimie* 58 (1976) 1409–1412.

- [69] O. Meilhac, B. Ho-Tin-Noe, X. Houard, et al., Pericellular plasmin induces smooth muscle cell anoikis, *FASEB J.* 17 (2003) 1301–1303.
- [70] A. Reijerkerk, L.O. Mosnier, O. Kranenburg, et al., Amyloid endostatin induces endothelial cell detachment by stimulation of the plasminogen activation system, *Mol. Cancer Res.* 1 (2003) 561–568.
- [71] A. Reijerkerk, E.E. Voest, M.F. Gebbink, No grip, no growth: the conceptual basis of excessive proteolysis in the treatment of cancer, *Eur. J. Cancer.* 36 (2000) 1695–1705.
- [72] S. Monea, K. Lehti, J. Keski-Oja, P. Mignatti, Plasmin activates pro-matrix metalloproteinase-2 with a membrane-type 1 matrix metalloproteinase-dependent mechanism, *J. Cell. Physiol.* 192 (2002) 160–170.
- [73] Y. Okumura, H. Sato, M. Seiki, H. Kido, Proteolytic activation of the precursor of membrane type 1 matrix metalloproteinase by human plasmin, A possible cell surface activator, *FEBS Lett.* 402 (1997) 181–184.
- [74] E.N. Baramova, K. Bajou, A. Remacle, et al., Involvement of PA/plasmin system in the processing of pro-MMP-9 and in the second step of pro-MMP-2 activation, *FEBS Lett.* 405 (1997) 157–162.
- [75] A. Takano, A. Hirata, Y. Inomata, et al., Intravitreal plasmin injection activates endogenous matrix metalloproteinase-2 in rabbit and human vitreous, *Am. J. Ophthalmol.* (2005).
- [76] B. Levkau, R.D. Kenagy, A. Karsan, et al., Activation of metalloproteinases and their association with integrins: an auxiliary apoptotic pathway in human endothelial cells, *Cell Death Differ.* 9 (2002) 1360–1367.
- [77] G. De Petro, D. Tavian, A. Copeta, et al., Expression of urokinase-type plasminogen activator (u-PA), u-PA receptor, and tissue-type PA messenger RNAs in human hepatocellular carcinoma, *Cancer Res.* 58 (1998) 2234–2239.
- [78] M. Lindgren, M. Johansson, J. Sandstrom, et al., VEGF and tPA co-expressed in malignant glioma, *Acta Oncol.* 36 (1997) 615–618.
- [79] C. Hackel, B. Czerniak, A.G. Ayala, K. Radig, A. Roessner, Expression of plasminogen activators and plasminogen activator inhibitor 1 in dedifferentiated chondrosarcoma, *Cancer* 79 (1997) 53–58.
- [80] J.C. Gris, J.F. Schved, C. Marty-Double, J.M. Mauboussin, P. Balmes, Immunohistochemical study of tumor cell-associated plasminogen activators and plasminogen activator inhibitors in lung carcinomas, *Chest* 104 (1993) 8–13.
- [81] J. Yamashita, K. Inada, S. Yamashita, et al., Tissue-type plasminogen activator is involved in skeletal metastasis from human breast cancer, *Int. J. Clin. Lab. Res.* 21 (1992) 227–230.
- [82] J.H. de-Witte, C.G. Sweep, J.G. Klijn, et al., Prognostic value of tissue-type plasminogen activator (tPA) and its complex with the type-1 inhibitor (PAI-1) in breast cancer, *Br. J. Cancer* 80 (1999) 286–294.
- [83] N. Grebenshikov, A. Geurts-Moespot, H. De Witte, et al., A sensitive and robust assay for urokinase and tissue-type plasminogen activators (uPA and tPA) and their inhibitor type I (PAI-1) in breast tumor cytosols, *Int. J. Biol. Markers* 12 (1997) 6–14.
- [84] S.J. Kim, E. Shiba, T. Kobayashi, et al., Prognostic impact of urokinase-type plasminogen activator (PA), PA inhibitor type-1, and tissue-type PA antigen levels in node-negative breast cancer: a prospective study on multicenter basis, *Clin. Cancer Res.* 4 (1998) 177–182.
- [85] M.S. Pepper, N. Ferrara, L. Orci, R. Montesano, Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells, *Biochem. Biophys. Res. Commun.* 181 (1991) 902–906.