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Stimulation of tPA-dependent provisional extracellular fibrin matrix degradation by human recombinant soluble melanotransferrin

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Abstract

Tissue-type plasminogen activator (tPA) and its substrate plasminogen (Plg) are key components in the fibrinolytic system. We have recently demonstrated, that truncated human recombinant soluble melanotransferrin (sMTf) could stimulate the activation of Plg by urokinase plasminogen activator and inhibit angiogenesis. Since various angiogenesis inhibitors were shown to stimulate tPA-mediated plasminogen activation, we examined the effects of sMTf on tPA-dependent fibrinolysis. This study demonstrated that sMTf enhanced tPA-activation of Plg by 6-fold. sMTf also increased the release of [^{125}I]-fibrin fragments by tPA-activated plasmin. Moreover, we observed that the interaction of sMTf with Plg provoked a change in the fibrin clot structure by cleaving the fibrin α and β chains. Overall, the present study shows that sMTf modulates tPA-dependent fibrinolysis by modifying the clot structure. These results also suggest that sMTf properties could involve enhanced dissolution of the provisional extracellular fibrin matrix.

Keywords: Fibrin matrix; Melanotransferrin; Plasminogen

1. Introduction

Plasmin is a serine protease that dissolves fibrin clots. Tissue-type plasminogen activator (tPA) plays a key role in fibrinolysis because tPA converts inactive plasminogen (Plg) into enzymatically active plasmin [1]. Melanotransferrin (MTf) is a 97 kDa glycoprotein that shares substantial sequence similarity with human serum transferrin, human lactoferrin, and chicken transferrin [2]. MTf was first identified, in the early 1980s, on the surface of melanoma cells and this glycoprotein is used as a marker for melanoma cells [3]. More recently, it was reported that MTf mRNA is present in many normal human tissues [4]. MTf exists as both membrane-bound and soluble forms, depending on whether or not this glycoprotein possesses a glycosylphosphatidylinositol (GPI)-anchor that has been attached to the glycoprotein [5].

Because MTf possesses iron-binding properties, it was first proposed that MTf might be involved in iron transport [5]. However, MTf has been shown to play a minor role in the uptake of iron [6]. Recent studies have suggested that MTf could be involved in pathological and physiological processes, including Alzheimer's disease [7], chondrogenic differentiation [8] and transcytosis across the blood–brain barrier [9]. We have previously shown that membrane-bound MTf could bind and stimulate Plg activation at the cell surface [10]. In addition, we also reported that truncated human recombinant soluble melanotransferrin (sMTf) could catalyze the urokinase type activator uPA-mediated activation of plasmin and affect cell migration [11]. We also found that sMTf inhibited endothelial cell movement and tubulogenesis which are important events in angiogenesis [12].

The formation of a provisional extracellular fibrin matrix (PEFM) is an important step in cell migration. This occurs after vascular injury, during inflammation, and in tumors. These phenomenons induce the expression of tissue factor on the endothelial cells [13]. Tissue factor, which is not only present on

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stimulated endothelial cells but in the subendothelial matrix and on many tumor cells, triggers the formation of PEFM [14]. Fibrin and the other components of this extracellular matrix are involved in the regulation of cell proliferation and migration through interactions with adhesion molecules on cell surfaces [15].

Since the conversion of Plg to plasmin by tissue-type plasminogen activator (tPA) plays a role in fibrinolysis, we investigated whether sMTf could affect fibrinolysis of PEFM. The generation of plasmin as well as the release of [^{125}I]-fibrin fragments by tPA increased in the presence of sMTf. In addition, sMTf enhanced the tPA-dependent fibrinolysis of both fibrin clots and platelet-rich plasma (PRP) clots by tPA. Overall, the different experimental approaches indicated that sMTf stimulates Plg activation by tPA leading to an increase in the fibrinolysis of PEFM.

2. Materials and methods

2.1. Materials

Truncated human recombinant MTf (sMTf), which is produced by introducing a stop codon following the glycine residue at position 711 (27 C-terminal amino acids deletion), and L235 monoclonal antibody (mAb) were kindly provided by Biomarin Pharmaceutical (Novato, CA). Fibrinogen, thrombin and tPA were from Calbiochem (La Jolla, CA). The antibody directed against MTf (L235) was from American Type Culture Collection (Manassas, VA). Blood tubes were 3.2% citrate-treated Vacutainers® from Becton Dickinson (Franklin Lakes, NJ). Human factor XIII Fibrogammin® P (FXIII) was from Aventis (Marburg, Germany). Plg was from Technoclone (Vienna, Austria). Other biochemical reagents were from Sigma (Oakville, ON).

2.2. Plasmin activity assay

Human glu-Plg (50 nM) (Technoclone, Vienna, Austria) activation by human tPA (50 pM) with or without sMTf (500 nM) (Calbiochem, La Jolla, CA) was measured using a colorimetric assay as previously described [16]. Briefly, the reaction was performed in a final volume of 200 μl in a suitable medium (buffer A, consisting of 50 mM Tris/HCl pH 7.4, 150 mM NaCl, and 50 mM $CaCl_2$). The reaction was started by the addition of tPA. The plasmin produced cleaved the plasmin substrate (Val-Leu-Lys-*p*-nitroanilide) (Sigma ,Oakville, ON). Absorbance was monitored at 405 nm using a Microplate Thermomax Autoreader (Molecular Devices, Sunnyvale, CA). The L235 mAb (2 μM) (American Type Culture Collection, Manassas, VA) was used to inhibit sMTf activity. Concentration variation of sMTf (0–10 μM) determined the apparent K_m to enhance tPa activation of Plg. K_m was evaluated using the Prism software (GraphPad Software Inc, San Diego, CA).

2.3. BIAcore analysis

Glu-Plg (3 μg) was covalently coupled to a sensor chip (CM5) via primary amine groups using the coupling reagents (*N*-hydroxysuccinimide (NHS)/ *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide (EDC)) as previously described [17]. sMTf was produced following an established protocol [18]. Proteins were injected onto the biological sensor chip surface. The surface plasmon resonance (SPR) generated by the protein–protein interaction was monitored in real-time and analyzed with BIAevaluation software (BIAcore, Piscataway, NJ) to determine the kinetic parameters of interaction.

2.4. Platelet-rich plasma (PRP) preparation

Human blood samples were collected by a two syringe technique, during which the first ml was discarded, of blood into 3.2% citrate-treated Vacutainers® (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 300×*g* for 5 min at room temperature. Participating subjects had given informed consent in accordance with the Declaration of Helsinki.

2.5. Fibrin plate assay

To examine the effects of sMTf on fibrinolysis, we used a [^{125}I]-fibrin plate assay as previously described [19]. Briefly, in a 24-well microplate, 20 μl of human, labeled [^{125}I]-fibrinogen (Amersham Biosciences, Bucks, UK) (6 nCi/assay) at 3 mg/ml were mixed with Plg (2 μM) and introduced into the wells. Clotting was achieved by the addition of human thrombin (Sigma, Oakville, ON) (0.4 U/ml) with or without human factor XIII Fibrogammin® P (FXIII) (Aventis, Marburg, Germany) for 60 min at 37 °C. Clots were carefully washed three times with the buffer A. Next, the buffer with tPA were carefully layered onto the surface of the clot and treated with sMTf (500 μM). The release of [^{125}I]-fibrin fragments into the supernatant (100 μl) during a 15 min incubation at 37 °C was measured by a LKB Wallac 1282 Compugamma counter (LKB Instruments, Inc, Gaithersberg, MD).

2.6. PRP fibrinolysis assay

To examine the influence of sMTf on fibrinolysis *ex vivo*, we used a [^{125}I]-fibrinogen labeled PRP clot assay. Labeled [^{125}I]-fibrinogen (6 nCi/assay) was mixed with 20 μl of PRP. Clotting was achieved by the addition of $CaCl_2$ (20 mM final) for 60 min at 37 °C. Next, 100 μl of buffer A containing tPA and various concentrations of sMTf was layered onto the surface of the clot. The release of [^{125}I]-fibrin fragments into the supernatant during a 15 min incubation at 37 °C was measured.

2.7. Radial clot lysis assay

To visualize the enhanced fibrinolysis due to sMTf , radial clot lysis was performed as previously described by Mosesson [16], with minor modifications. Briefly, fibrin clots were obtained by incubating fibrinogen (8.2 μM), Glu-Plg (2 μM) and 0.4 U/ml thrombin in buffer A at 37 °C for 60 min in a 6-well plate. Clot lysis was initiated by adding 2 μl of tPA (1 nM) with or without sMTf (100 nM). Clots were incubated for 30 min at 37 °C and dyed with Chinese ink. Photomicrographs at 40× magnification were taken using a Nikon Coolpix 5000 digital camera attached to a Nikon TMS-F microscope (Nikon Canada, Mississauga, ON).

2.8. Cleavage of fibrin clot by sMTf

To visualize the influence of hr-sMTf on the fibrin clot, we incubated fibrinogen, Plg and thrombin at 37 °C for one h. After the clot had polymerized, the clot was treated with hr-sMTf for 8 h at 37 °C. The clot was then dissolved under reducing conditions [20]. Electrophoresis was carried out on a 9% acrylamide gel at 100 V for 2 h. The gel was dyed afterwards with Coomassie blue. Protein sequencing was performed by NRC Protein and Peptide Sequencing (Montreal, QC).

2.9. Thromboelastography analysis

Thromboelastography analysis was performed with PRP or with an artificial clot model using a computerized dual-channel thromboelastograph (TEG) analyzer (model 5000; Haemoscope Corp., Niles, IL). For the artificial clot model, fibrinogen (8.2 μM) (Sigma, Oakville, ON), Glu-Plg (3.3 μM) and tPA (1 nM) were diluted in buffer A and transferred into the analyzer cups. Artificial clots were polymerized with thrombin (Sigma, Oakville, ON) (0.4 U/ml). For the PRP clots, 350 μl of PRP were transferred into the analyzer cups with tPA (1 nM). $CaCl_2$ (0.2 M) was added to initiate the coagulation of PRP. Thromboelastography analyses for both artificial clot models and PRP clots were performed in both the presence and absence of 1 μM sMTf at 37 °C.

2.10. Data analysis

Statistical analyses were performed using Student's t-test via GraphPad Prism software (San Diego, CA). Significant difference was assumed for *P*

values less than 0.05. The measurement of variation is reported as the mean± standard deviation, measured in triplicate.

3. Results

3.1. Plasmin activity assay

In order to characterize the effect of sMTf on tPA activation of Plg, the plasmin activity assay was evaluated by the hydrolysis of VLK *p*-nitroanilide, a colorimetric substrate of plasmin (Fig. 1). After 3 h of incubation in the presence of Plg, sMTf increased the tPA-mediated generation of plasmin by 6-fold (●), compared to tPA alone (○). sMTf with Plg had no proteolytic or plasmin-like activity itself (■) (Fig. 1a). The specificity of the effect of sMTf was then determined by measuring the induction of tPA-dependent Plg activation by sMTf in the presence of either mAb L235 (○), an antibody directed against sMTf, or a non-specific IgG used as a control (●). The L235 mAb (2 μM) inhibited the effect of sMTf on tPA-induced plasmin generation by 80% (Fig. 1b). To determine the optimal concentration of sMTf needed to enhance the activation of Plg by tPA, the half-maximal concentration was determined. sMTf stimulated the initial rate of tPA-dependent conversion of Plg into plasmin in a dose-dependent manner with a half-maximal stimulation occurring at 53±22 nM (Fig. 1c). The effect of sMTf on plasmin formation by tPA was further evaluated in the presence of various concentrations of Plg. Initial rates of plasmin activity indicated that sMTf decreased the apparent K_m of tPA for Plg from 280 nM (○) to 52 nM (●) (Fig. 1d).

3.2. BIAcore analysis

The ability of sMTf to interact with Plg was monitored in real-time using a BIAcore apparatus. Standard NHS/EDC coupling procedures [17] permitted immobilization of Plg on the BIAcore sensor chip surface. Injections of sMTf over immobilized Plg generated SPR, which increased in an sMTf concentration-dependent fashion (Fig. 2). Using this approach, the apparent equilibrium dissociation constant K_D, calculated with the Biaevaluation software, was 260 nM. The best fit used was the two state reaction (conformation change).

3.3. Fibrin plate assay

We next measured the release of [^{125}I]-fibrin fragments from labeled clots after adding with Plg at a physiological concentration to determine the effect of sMTf on fibrinolysis (Fig. 3). sMTf increased the release of [^{125}I]-fibrin fragments by 2.5 fold following Plg activation by tPA (Fig. 3a). The release of [^{125}I]-fibrin fragments was next measured from clots produced in the presence of factor XIII (Fig. 3b) and showed that a higher concentration of tPA was needed to release the same amount of [^{125}I]-fibrin fragments as was released in the

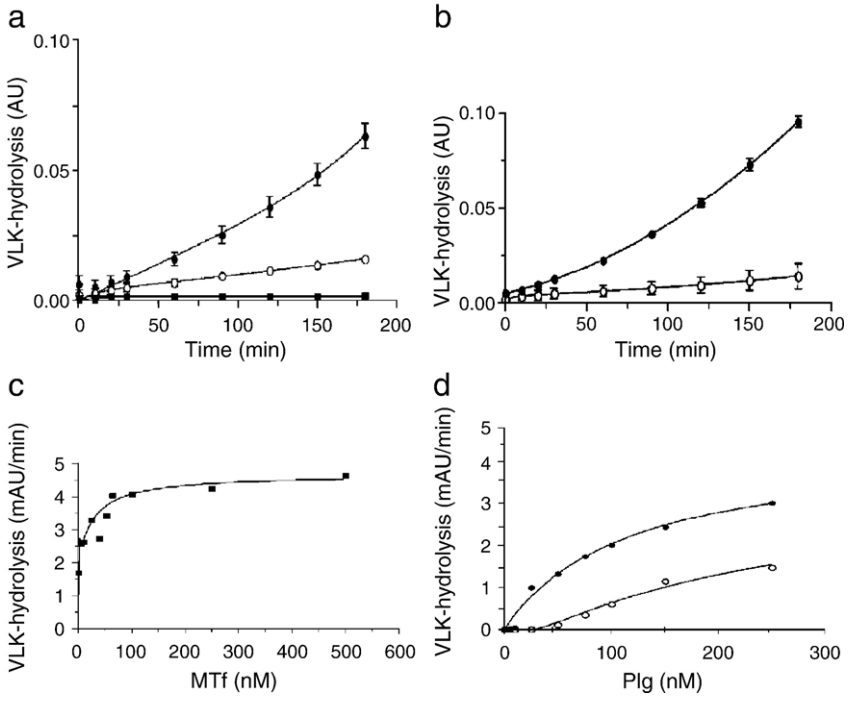

Fig. 1. Effects of sMTf on tPA-dependent plasmin activity. (a) The plasmin activity induced by tPA was measured without (○) or with sMTf (●) in the presence of Plg. The reaction was performed as described in Materials and methods. The plasminolytic activity in the presence of sMTf (■) alone was also measured. Data shown are based upon 3 independent experiments. *P< 0.05 vs. tPA. (b) The plasmin activity induced by tPA was measured in the presence of sMTf with either the mAb L235 (○) or a non-specific mouse IgG (●). (c) Plasmin activity induced by tPA was determined by measuring VLK-hydrolysis in the presence of various sMTf concentrations. (d) Initial rates of VLK-hydrolysis during Plg activation by tPA were measured without (○) or with (●) sMTf in the presence of various concentrations of Plg. Data shown are means±SD of 3 different experiments.

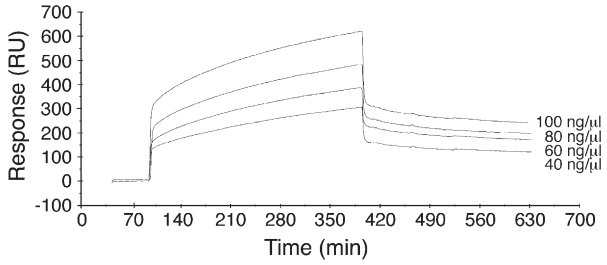

Fig. 2. Biospecific interaction analysis in real-time between sMTf and Plg. sMTf diluted in Buffer A was injected onto immobilized Plg on a sensor chip as in Materials and methods. The SPR response for these proteins was plotted in response units (RU) as a function of time.

absence of factor XIII. sMTf increased the rate of [^{125}I]-fibrin fragment release from PRP clots in the presence of tPA (Fig. 4). In this assay system, the apparent concentration of sMTf needed to support a 50% maximal [^{125}I]-fibrin fragment released by tPA is 96 nM.

3.4. Radial clot lysis assay

To further demonstrate the effect of sMTf on tPA-induced clot lysis, a radial clot lysis assay was used. (Fig. 5). The addition of sMTf to tPA enhanced fibrinolysis, leading to increased dissolution of the fibrin clot (Fig. 5a). In the absence of tPA, the addition of sMTf to Plg modified the fibrin clot structure, whereas sMTf in the absence of Plg did not seem to affect it. (Fig. 5b).

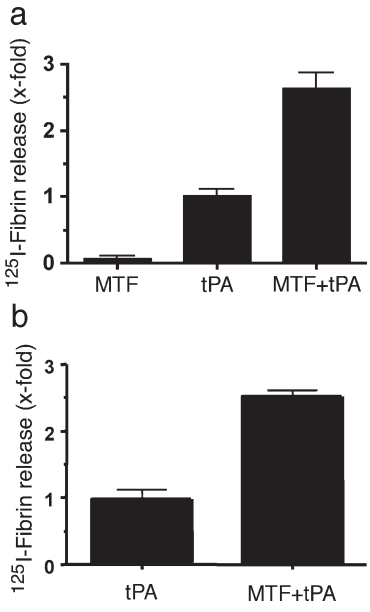

Fig. 3. Impact of sMTf on tPA-dependent fibrinolysis. (a) Release of [^{125}I]-fibrin fragments was measured in the presence of sMTf, tPA (50 pM) or both sMTf and tPA, and was quantified using a gamma scintillation counter. Relative release was established as 1-fold for tPA. Data shown (means and SD) are from 8 experiments. **$P<0.002$ vs. baseline. ***$P<0.001$ vs. tPA (b) Fibrin release was compared between clots which had been crosslinked with FXIII (1U/ml) using two different conditions, tPA (1 nM) and tPA + sMTf (500 nM). ***$P<0.001$ vs. tPA. Data shown (means and SD) are from 8 experiments.

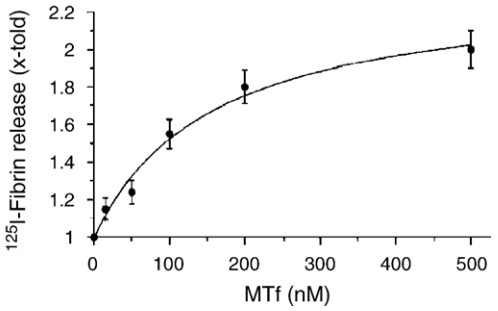

Fig. 4. Modulation of tPA-dependent fibrinolysis of PRP clot by sMTf. Release of [^{125}I]-fibrin fragments was measured in PRP containing tPA with varying concentrations of sMTf. The data shown are from 3 independent experiments. Values are means ± SD. *$P<0.05$ vs. tPA.

3.5. Evaluation of fibrin fragmentation

The fibrin fragmentation pattern was next analyzed in the absence of tPA to further investigate the effects of sMTf on the fibrin clot structure. The fragmentation pattern was obtained by measuring the migration of treated fibrin clots on acrylamide gels by electrophoresis under reducing conditions (Fig. 6). Electrophoresis performed in the presence of the reducing agent β-mercaptoethanol allowed visualization of the 2 chains of fibrin (α, β). The addition of either sMTf or Plg did not affect the electrophoresis separation profiles. However,

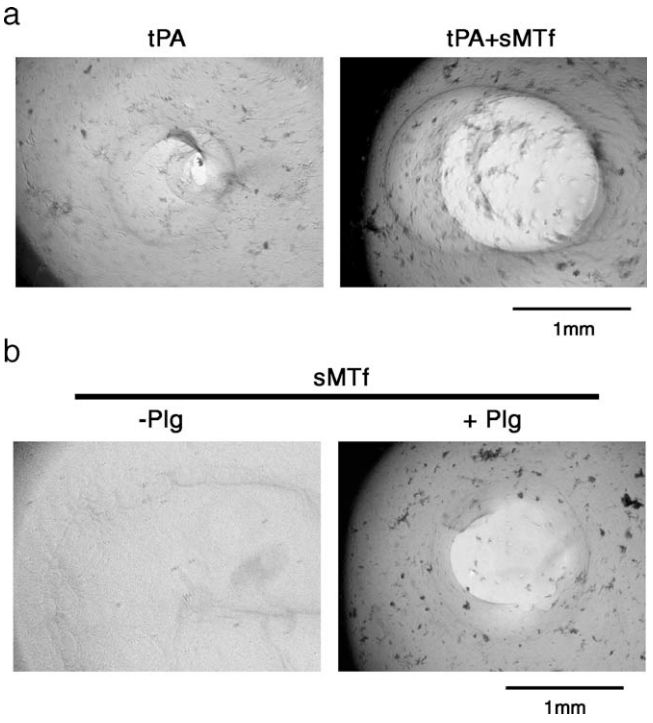

Fig. 5. Effect of sMTf on clot fibrinolysis with tPA. (a) Human fibrinogen was clotted with thrombin in the presence of Plg in a 6 well plate. In each well, a 2 µl volume of either tPA, tPA + sMTf or sMTf alone was laid down onto the fibrin clot. Clots were dyed with Chinese ink and digitally measured. Experiments shown are representative of multiple independent experiments. (b) The fibrin clot was treated with sMTf in the presence or absence of Plg. Photographs were taken at 100× magnification.

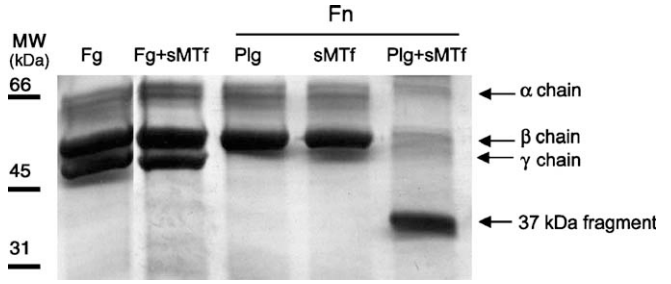

Fig. 6. Degradation of human fibrin (Fn). Human fibrinogen (Fg), Plg and thrombin were incubated at 37 °C for one h. After the clot had polymerized, the clot was treated with sMTf for 8 h at 37 °C. The clot was then dissolved under reducing conditions [20]. Electrophoresis was carried out on a 9% acrylamide gel at 100 V for 2 h. The gel was dyed afterwards with Coomassie blue. Protein sequencing was performed to determine the site of cleavage.

the incubation of fibrin with both sMTf and Plg strongly altered the separation profile. Both chains (α and β) of fibrin almost completely disappeared, leaving a fragment of 37 kDa. Peptide sequencing of the 37 kDa fragment N-terminal region produced the sequence DVENV, which represents the region between Asp^{165} and Val^{169} of the fibrin β-chain.

3.6. Evaluation of clot viscoelastic properties

Since MTf potentiates Plg activation by tPA, the impact of MTf on clot formation and lysis was therefore evaluated using TEG analysis (Fig. 7). An artificial fibrin-clot model formed by the action of thrombin on fibrinogen was first used (Fig. 7a). This model allowed examination of the effect of MTf on tPA-fibrinolysis in the absence of inhibitors. Monitoring of the TEG parameters indicated that addition of MTf increased the thrombolytic activity of tPA (Table 1). In particular, when MTf (500 nM) was added to tPA, clot lysis after 30 min (LY30) was 5 times higher than that observed in the absence of MTf while the complete lysis time (CLT) diminished by 30%. The impact of MTf on fibrin-clot dissolution was further evaluated using PRP (Fig. 7b). The TEG parameters obtained for these experiments (Table 1) indicated that the addition of MTf to tPA caused a 30% decrease in the maximum clot strength (MA), doubled the LY30 rate and reduced the CLT by 20%.

4. Discussion

Previous studies showed that various angiogenesis inhibitors stimulate tPA-mediated plasminogen activation [21–23]. The plasminogen activation system, which leads to the formation of the serine protease plasmin and to subsequent fibrinolysis, has been shown to play an important role in the breakdown of the PEFM, one of the hallmark of angiogenesis [24]. We report here that sMTf enhances the formation of plasmin by tPA by interacting with Plg. In haemostasis, tPA is predominantly secreted by endothelial cells and cleaves circulating Plg into plasmin which is the enzyme responsible for proteolytic degradation of fibrin [19]. The apparent K_m for Plg cleavage by tPA was similar to that measured in a previous study [22]. The action of sMTf on Plg reduced the K_m for tPA by 5 fold.

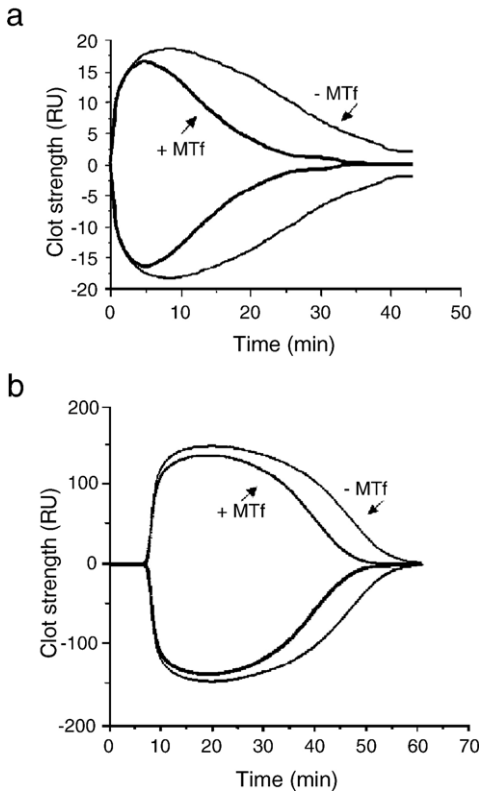

Fig. 7. Effect of MTf on clot strength and fibrinolysis. Representative tracing showing effects of MTf (500 nM) on clot strength in relative units (RU) during the fibrinolysis of clot formation under shear by TEG. (a) Thromboelastogram of the fibrin clot model. (b) Thromboelastogram of PRP clotted after addition of $CaCl_2$ (2 mM). The results shown here are representative of 3 different experiments.

Inhibition by the L235 mAb of the ability of sMTf to induce the activation of Plg by tPA suggests that its interaction with Plg is specific and might involve the conformational epitope recognized by this mAb [25]. The ability of the anti-MTf antibody to block this inductive effect confirms that the biologically active factor was sMTf itself rather than a contaminating factor. The interaction of sMTf with immobilized Plg confirmed results from a previous study, where we showed that Plg could interact

Table 1
Effect of MTf on thromboelastograph parameters

Parameters	Conditions	
	tPA	tPA + MTf
(a) Artificial fibrin-clot		
(1) MA	498±7	446±17
(2) LY30 %	25.0±8.2	72.5±20.4
(3) CLT min	54.3±10.0	37.8±3.8
(b) Fibrin-clot with PRP		
(1) MA	13465±1586	9560±1626
(2) LY30 %	4.3±0.7	11.8±4.0
(3) CLT min	68.3±1.6	49.1±6.3

The three parameters obtained with the thromboelastograph are presented as follows: 1. MA is the maximum strength of the clot at maximum amplitude of the TEG trace., 2. The percentage of lysis at 30 min (LY 30). 3. The complete clot lysis time in minutes (CLT).

with immobilized sMTf [11]. The K_D values were similar in both studies. Several reports have established that protein–protein interactions can positively modulate the activity of an enzyme [26]. Thus, sMTf can be viewed as a positive modulator of tPA-dependent Plg activation and dose response studies have revealed that sMTf is active in the nM range. sMTf modulates the tPA-induced release of [^{125}I]-fibrin fragments from artificial clots and from PRP clots. Although the introduction of factor XIII augments resistance to tPA-induced fibrinolysis [27], the addition of sMTf still increased the release of [^{125}I]-fibrin fragments. The clot assay provides another line of evidence that the activation of Plg by tPA is increased by sMTf and leads to enhanced dissolution of the PEFM. Fibrin is not strictly required for tumor angiogenesis: however, the absence of fibrin in a knock-out model strongly diminishes the aggressiveness of the tumor [28].

Previous studies have established a clear link between modification of the fibrin clot structure, allowing better penetration of tPA, and acceleration of tPA-dependent clot dissolution [29]. These observations are supported by the degradation pattern of fibrin in the presence of Plg and sMTf, which demonstrated a cleavage of the fibrin β chain and the degradation of the α chain in the absence of tPA. γ chain under these conditions forms dimers (≅100 kDa) that migrate in the same region of sMTf and Plg [30]. The cleavage of the β chain in the presence of sMTf would thus increase the accessibility of Plg for tPA. Since a higher rate of clot lysis is observed with the increased access of Plg [31], increased accessibility of Plg by sMTf could greatly facilitate the action of tPA. Moreover, the lysis of composite fibrin fibres initiated by either plasmin or tPA proceeds preferentially by lateral section of fibers, rather than by uniform thinning of the whole fibre [32]. This change in fibrin architecture is also visible by the diminished strength and accelerated fibrinolysis of the clot. The cleavage of the α and β chains would fragment the lateral section released and accelerate the degradation of the fibrin. This phenomenon could prevent the exposition of binding sites for anchor receptors of endothelium cells or tumor cells in the PEFM [33].

In conclusion, these first results clearly demonstrated that sMTf catalyzes the activation of Plg by tPA, leading to enhanced tPA-mediated fibrinolysis. Thus, sMTf affects the activation of Plg by both activators: urokinase [10–12] and tPA. Our data demonstrate that sMTf, in combination with Plg in the PEFM, modifies the fibrin clot structure. These are the first results suggesting that administration of sMTf could impair the PEFM needed both by the endothelium to produce new capillary networks and by tumor cells to migrate. Further studies are underway to elucidate whether endogenous MTf could be related to fibrinolysis-associated events like vascular disease.

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