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Inhibition of endothelial cell movement and tubulogenesis by human recombinant soluble melanotransferrin: involvement of the u-PAR/LRP plasminolytic system

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Abstract

We have previously demonstrated that human recombinant soluble melanotransferrin (hr-sMTf) interacts with the single-chain zymogen pro urokinase-type plasminogen activator (scu-PA) and plasminogen. In the present work, the impact of exogenous hr-sMTf on endothelial cells (EC) migration and morphogenic differentiation into capillary-like structures (tubulogenesis) was assessed. hr-sMTF at 10 nM inhibited by 50% the migration and tubulogenesis of human microvessel EC (HMEC-1). In addition, in hr-sMTf-treated HMEC-1, the expression of both urokinase-type plasminogen activator receptor (u-PAR) and low-density lipoprotein receptor-related protein (LRP) are down-regulated. However, fluorescence-activated cell sorting analysis revealed a 25% increase in cell surface u-PAR in hr-sMTf-treated HMEC-1, whereas the binding of the urokinase-type plasminogen activator (u-PA)•plasminogen activator inhibitor-1 (PAI-1) complex is decreased. This reduced u-PA-PAI-1 binding is correlated with a strong inhibition of the HMEC-1 plasminolytic activity, indicating that exogenous hr-sMTf treatment alters the internalization and recycling processes of free and active u-PAR at the cellular surface. Overall, these results demonstrate that exogenous hr-sMTf affects plasminogen activation at the cell surface, thus leading to the inhibition of EC movement and tubulogenesis. These results are the first to consider the potential use of hr-sMTf as a possible therapeutic agent in angiogenesis-related pathologies.

Keywords: Melanotransferrin; Urokinase-type plasminogen activator receptor; Plasminogen; Cell movement; Tubulogenesis

1. Introduction

Melanotransferrin (MTf) is a glycosylated protein that was first identified in the early 1980s as human melanoma antigen p97 since it was found at high levels in malignant melanoma cells [1,2]. Because of its homology to transferrin [2], it was later called melanotransferrin [3]. Slightly expressed in normal tissues, MTf was found in much larger amounts in neoplastic cells and fetal tissues [1,4,5]. More recently, there have been additional reports of human MTf being detected in sweat gland ducts, salivary glands, liver endothelial cells (EC), brain endothelium and chondrocytes [6–9]. Two forms of MTf have been described to date. MTf can be secreted in a soluble form (sMTf) or remains bound to the cell membrane by a glycosyl phosphatidylinositol anchor, which can be cleaved by phospholipase C [7,10,11]. It was first thought that MTf could serve as an iron transporter; however, it was later shown that MTf played very little role in iron transport [12–14]. In addition, a recent study demonstrated that sMTf inefficiently donates iron to cells [15]. Interestingly, normal serum contains very low levels of circulating sMTf, which were reported to increase by five- to sixfold in patients with Alzheimer's disease [16–18]. Thus, it has been suggest that sMTf serum levels was a candidate biochemical marker of

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Alzheimer's disease. Nevertheless, a recent study shows that serum sMTf levels remain constant in subject with Alzheimer's disease compared to healthy individual [19]. The physiological roles of both forms of MTf are still unclear.

Angiogenesis, a complex multistep process that leads to the outgrowth of new capillaries from pre-existing vessels, is an essential mechanism in wound healing, embryonic development, tissue remodelling and in tumor growth and metastasis [20,21]. This process involves EC proliferation, migration and morphogenic differentiation into capillary-like structures [22]. One of the key elements in cell migration is the urokinase-type plasminogen activator receptor (u-PAR). The plasminogen activator (PA) family is composed of urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA); their inhibitors are the plasminogen activator inhibitor types 1 and 2 (PAI-1; PAI-2). u-PAR mediates the internalization and degradation of u-PA/inhibitor complexes via the low-density lipoprotein receptor-related protein (LRP) [23–27], whereas LRP mediates the internalization and degradation of t-PA/inhibitor complexes [28]. Thus, the u-PAR/LRP plasminolytic system controls cell migration by regulating plasminogen activation by PAs at the cell surface [29–33]. PAs are therefore involved in angiogenesis, where localized proteolysis is required, by stimulating fibrinolysis as well as cell migration and invasion [34,35]. When Glu-plasminogen, the native circulating form of the zymogen, is bound to the cell surface, plasmin generation by PAs is markedly stimulated compared with the reaction in solution [36]. Several studies have shown that plasmin, a proangiogenic proteinase fragment released from plasminogen, promotes cell migration and angiogenesis when activated at the cell surface [36–40].

Since human recombinant soluble melanotransferrin (hr-sMTf) interacts with the single-chain zymogen pro u-PA (scu-PA) and plasminogen [41], we investigated the potential effect of hr-sMTf on tubulogenesis. Here, we show that hr-sMTf inhibits EC movement and tubulogenesis. Because LRP and u-PAR are key receptors in these two processes, we measured the expression of both LRP and u-PAR in human microvessel EC (HMEC-1) cell lysates and at their cellular surface. In addition, to determine the complexation state of uPAR at the cell surface of hr-sMTf-treated HMEC-1, we next measured the binding of the uPA•PAI-1 complex. The present study indicates that hr-sMTf inhibits EC movement and tubulogenesis by altering the internalization and recycling processes of free and active u-PAR at the cellular surface.

2. Materials and methods

2.1. Materials

Human recombinant sMTf (hr-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). Antibodies directed against α-LRP (8G1 clone) and u-PAR (#3937) were from Research Diagnostics Inc. (Flanders, NJ) and American Diagnostica (Greenwich, CT), respectively. The antibody directed against GAPDH (#RGM2) was from Advanced Immunochemical Inc. (Long Beach, CA). Antibodies directed against extracellular signal-regulated kinase 1/2 (ERK 1/2) (#9102) and pERK 1/2 (#9101S) were from Cell Signaling Technology (Beverly, MA). u-PA and PAI-1 were from American Diagnostica. Other biochemical reagents were from Sigma (Oakville, ON).

2.2. Cell culture

Cells were cultured under 5% CO_2/95% air atmosphere. HMEC-1 cells were from the Center for Disease Control and Prevention (Atlanta, GA) and were cultured in MCDB 131 medium (Sigma) containing 10 mM L-glutamine, 10 ng/mL epidermal growth factor (EGF), 1 μg/mL hydrocortisone and 10% inactivated fetal bovine serum (FBS). Human umbilical vessel EC (HUVEC) were obtained from ATCC (Manassas, VA). HUVEC were cultured in EGM-2 medium (Bullet kit, Clonetics #CC-3162) containing 20% inactivated FBS.

2.3. Plasminolytic activity assay

The in vitro enzymatic activity of scu-PA and u-PA was measured using colorimetric assay with or without hr-sMTf (70 nM). The reaction was performed in a final volume of 200 μL in an incubation medium consisting of 50 nM Tris/HCl buffer (pH 7.5), 150 nM NaCl and 50 mM $CaCl_2$. This incubation medium also contained 30 nM plasminogen and 15 μg of the chromogenic plasmin substrate D-Val-Leu-Arg *P*-Nitroanilide (VLK-pNA). The reaction was started by the addition of scu-PA or u-PA. In this assay, the cleavage of VLK-pNA results in a *P*-nitraniline molecule that absorbs at 405 nm. The product was monitored at 405 nm after 60-min incubation at 37 °C using a Microplate Thermomax Autoreader (Molecular Devices, Sunnyvale, CA).

HMEC-1 were grown to 85% confluence in six-well plates and were incubated for 18 h under 5% CO_2/95% air atmosphere in cell culture medium with or without exogenous hr-sMTf (100 nM). After cell treatment, HMEC-1 were individualized by phosphate buffer saline (PBS) citrate solution (138 mM NaCl, 2.8 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 15mM sodium citrate, pH 7.4) for 15 min. Cells were washed twice in Ringer/HEPES solution (150 mM NaCl, 5.2 mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 6 mM $NaHCO_3$, 5 mM HEPES, 2.8 mM glucose, pH 7.4) and counted. 1×10^5 cells was incubated in the plasminolytic assay with 30 nM plasminogen and 15 μg of VLK-pNA. Plasmin activity was monitored at 405 nm after 480-min incubation at 37 °C using a Microplate Thermomax Autoreader.

2.4. Cell movement assay

HMEC-1 cell movement was performed using transwell filters (Costar; 8-μm pore size) precoated with 0.15% gelatin as previously described [41]. Brefly, 1×10^4 cells were resuspended in 100 μL of fresh medium with or without exogenous hr-sMTf (10 or 100 nM; native or boiled for 30 min at 100 °C), aprotinin (1 μM) as well as ϵ-aminocaproic acid (EACA; 100 mM) and added into the upper chamber of each transwell (lower chamber of the transwell also contained exogenous hr-sMTf, aprotinin or EACA). The plates were then placed at 37 °C in 5% CO_2/95% air atmosphere for 18 h Cells that have passed to the lower surface of the filters were fixed with 3.7% formaldehyde in PBS and stained with 0.1% crystal violet/20% MeOH. Passed cells were also visualized at a 100× magnification using a digital Nikon Coolpix™ 5000 camera (Nikon Canada, Mississauga, ON) attached to a Nikon TMS-F microscope (Nikon Canada) and passed cells contained in five random field per well were counted.

2.5. Capillary-like structure formation (tubulogenesis) assay

Aliquots (50 μL) of Matrigel (BD Bioscience, Mississauga, ON) were added to a 96-well plate and incubated for 10 min at 37 °C. HMEC-1 or HUVEC cells were harvested by trypsinization. 2.5×10^4 cells were resuspended in 100 μL fresh medium and added to Matrigel-coated wells for 30 min at 37 °C. After cell adhesion, the medium was removed and 100 μL of fresh cell culture medium was added with or without exogenous hr-sMTf (1, 5, 10 or 100 nM). Cells were then incubated for 18 h at 37 °C. After incubation, tubular structures were visualized at a 40× magnification using a digital Nikon Coolpix™ 5000 camera attached to a Nikon TMS-F microscope. The length of the total capillary network was quantified using a map scale calculator by measuring and summing the length of all tubular structures observed in a random field.

2.6. Western blot analysis

HMEC-1 (3×10^6 cells) were plated into a 75-cm^2 culture flask and exposed to fresh cell culture medium with or without exogenous hr-sMTf (10 or 100 nM). After 18-h treatment, conditioned media was removed and the cells were solubilized in lysis buffer [1% Triton-X-100, 0.5% NP-40, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 2% *N*-octylglucoside, 1 mM orthovanadate, pH 7.5]. HMEC-1 cell lysates was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (PerkinElmer Life Sciences, Boston, MA). Following transfer, immunodetection analysis was performed. Protein expression levels were quantified by laser densitometry using Chemilmager™ 5500 from Alpha Innotech Corporation (San Leandro, CA).

2.7. Total RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

HMEC-1 (3×10^6 cells) were plated into a 75-cm^2 culture flask and exposed to fresh cell culture medium with or without exogenous hr-sMTf (10 or 100 nM). After 18-h treatment, total RNA was extracted from HMEC-1 using TRIzol reagent from Invitrogen (Burlington, ON) using standard procedure. RT-PCR reactions were performed using SuperScript™ One-Step RT-PCR from Invitrogen. Specific primers used in this study were determined using MacVector™ software and were based on human cDNA sequences deposit at the NCBI data bank (LRP, #NM_002332; LRP1B, #NM_018557; LRP2, #NM_004525; LDL-R, #NM_000527; LRP5, #NM_002335; LRP8, #NM_033300; GAPDH, #BC 013852). The upstream and downstream primers used were as follows: for u-PAR: 5′-ACCGAGGTTGTGTGTGGGTTAGAC-3′ and 5′-CAGGAAGTGGAAGGTGTCGTTG-3′ (expected product 306 bp); for LRP: 5′-AGAAGTAGCAGGACCAGAGGG-3′ and 5′-TCAGTACCCAGGCAGTTATGC-3′ (expected product 301 bp); for LRP 1B: 5′-TCTCTCCTTCTCCAAAGACCC-3′ and 5′-TCAATGAGTCCAGCCAGTCAGC-3′ (expected product 403 bp); for LRP 2: 5′-CGGAGCAGTGTGGCTTATTTTC-3′ and 5′-CAGGTGTATTGGGTGTCAAGGC-3′ (expected product 180 bp); for LDL-R: 5′-GGACCCAACAAGTTCAAGTGTCAC and 5′-AAGAAGAGGTAGGCGATGGAGC-3′ (expected product 377 bp); for LRP 5: 5′-GCCATCAAGCAGACCTACCTGAAC-3′ and 5′-GCCAGTAAATGTCCGAGTCCAC-3′(expected product 334 bp); for LRP 8: 5′-CCTTGAAGATGATGGACTACCCTCG-3′ and 5′-AAAACCCAAAAAAGCCCCCCCAGC-3′ (expected product 415 bp); for GAPDH: 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTG-3′ (expected product 540 bp). RT-PCR conditions were optimized so that the gene products were obtained during the exponential phase of the amplification. Gene product amplification was performed for 40 cycles of PCR (94 °C for 15 s, 60 °C for 30 s (55 °C for LRP 2), 72 °C for 1 min) and amplification products were fractionated on 2% (w/v) agarose gels and visualized by ethidium bromide.

2.8. ^{125}I-uPA•PAI-1 complex binding

u-PA was radioiodinated using standard procedures with Na-^{125}I (Amersham Pharmacia Biotech, Baie D'Urfé, QC) and an iodo-beads kit from Pierce (Rockford, IL). ^{125}I-uPA•PAI-1 complexes were then generated as previously described [25]. First, HMEC-1 was treated or not with hr-sMTf (100 nM) for 18 h. Binding experiments were performed at 4 °C to limit potential internalization processes during the binding assay time frame. Following cell treat-

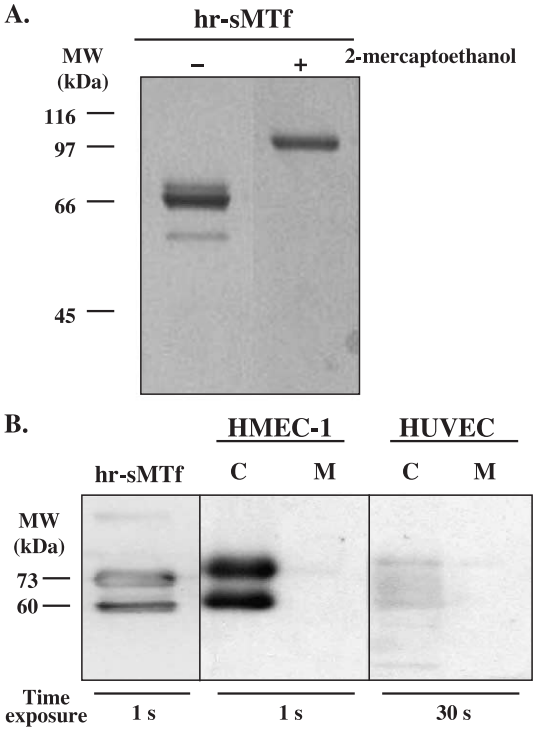

Fig. 1. MTf expression in endothelial cells. (A) hr-sMTf SDS-PAGE analysis. hr-sMTf (10 ng) was dissolved in Laemmli buffer in the absence (−) or the presence (+) of 5% (v/v) 2-mercaptoethanol and analyzed by SDS-PAGE. hr-sMTf was detected by Coomassie blue staining. (B) Immunodetection of hr-sMTf and endogenous MTf by Western blotting. Endogenous MTf was immunodetected in cell lysates (C) or serum-deprived conditioned culture media (M) from HMEC-1 and HUVEC cells. Proteins were separated by SDS-PAGE and were transferred to PVDF membranes. MTf was immunodetected using mAb L235 and a secondary antimouse IgG linked to peroxidase. One representative experiments is shown (n=3).

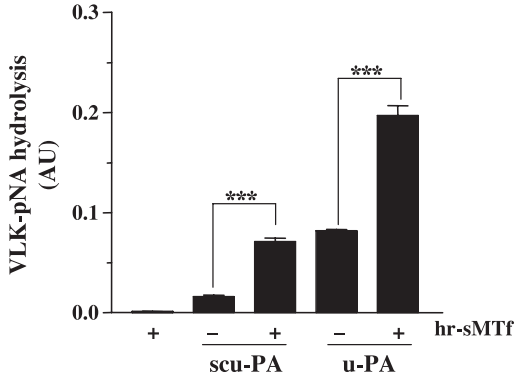

Fig. 2. hr-sMTf stimulates in vitro plasminogen activation. The activation of plasminogen by 1 nM scu-PA or u-PA was measured with or without 70 nM hr-sMTf. The reaction was performed in a final volume of 200 μL as described in Materials and methods. As a control, the enzymatic activity in the presence of hr-sMTf alone was also measured. Data represent the means±SD of three independent experiments performed in triplicates. Statistically significant differences are indicated by ***P<0.001 (Student's t test).

ment, the binding was initiated by adding 10 nM of ^{125}I-uPA•PAI-1 complex in Ringer/HEPES containing 0.05% ovalbumine. After 1-h incubation, cells were washed and lysed in NaOH (0.3 M). Cell associated radioactivity was quantified after trichloroacetic acid (TCA) precipitation.

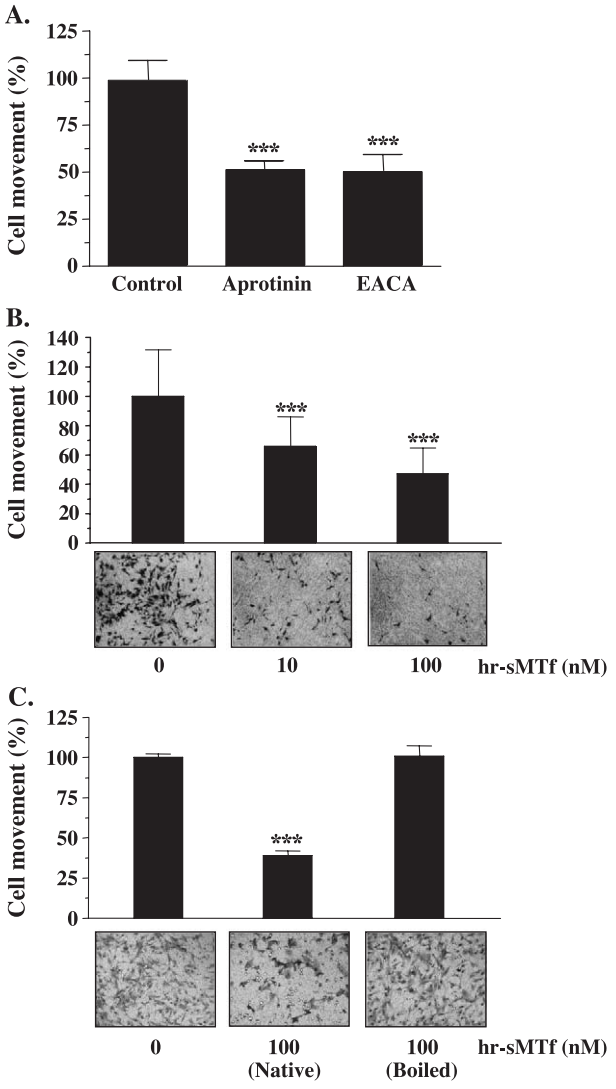

Fig. 3. Requirement of hr-sMTf native conformation in the inhibition of HMEC-1 cell movement. HMEC-1 cell movement was performed using modified Boyden chambers as described in the Materials and methods. Cells that had passed to the lower surface of the filters were fixed, stained with crystal violet and counted. (A) HMEC-1 cell movement was performed in the presence or absence of plasmin inhibitors, aprotinin and EACA. Results are expressed as a percentage of cell movement in inhibitor-treated cells compared to untreated cells. (B) HMEC-1 cell movement was performed in the presence or absence of hr-sMTf. Results are expressed as a percentage of movement in hr-sMTf-treated cells compared to untreated cells. Data represent the means±SD of four independent experiments performed in triplicates. (C) HMEC-1 cell movement was performed with native or boiled hr-sMTf. Results are expressed as a percentage of cell movement of hr-sMTf-treated cells compared to untreated cells. Data represent the means±SD of two independent experiments performed in triplicates. Statistically significant differences with control values are indicated by ***P<0.001 (Student's t test). Photos (original magnification, ×100) obtained from a representative experiments are shown.

2.9. Fluorescence-activated cell sorting (FACS) analysis

HMEC-1 (3×10^6 cells) were plated onto 75-cm^2 dishes using fresh media supplemented or not with hr-sMTf (100 nM). After 18-h incubation, HMEC-1 cells were individualized by incubation with PBS–citrate solution. HMEC-1 (1×10^6 cells) were resuspended in the binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and incubated at 4 °C for 15 min with 1 μg/mL of either u-PAR antibody, α-LRP antibody or with a nonspecific control IgG1. The cells were then washed twice with binding buffer and incubated in the dark at 4 °C for 15 min with 1 μg/mL goat anti-mouse IgG-Alexa488 (Molecular Probes, Eugene, OR). After two washes with binding buffer, the cells were analyzed by flow cytometry on a Becton Dickinson FACscan with a 488-nm Argon laser. Cell surface expression levels of u-PAR and α-LRP were corrected for the background fluorescence intensity measured in the presence of a nonspecific IgG1 and were expressed as mean fluorescence intensities.

2.10. Data analysis

Statistical analyses were made with Student's paired t-test using GraphPad Prism (San Diego, USA). Significance was assumed for P values less than 0.05.

3. Results

3.1. MTf expression in EC

We first characterized the hr-sMTf by SDS-PAGE and endogenous MTf expression in EC by Western blot (Fig. 1). Under nonreducing and denaturing conditions, hr-sMTf migrates as a 60- and 73-kDa protein (Fig. 1A) whereas under reducing and denaturing conditions, hr-sMTf migrates as a single band at 97 kDa (Fig. 1A). In addition, hr-sMTf migrates at a slightly lower molecular weight as endogenous MTf, which is mostly detected in HMEC-1 lysate by the L235 mAb (Fig. 1B). However, MTf is almost undetectable in both cell lysates and conditioned culture media from HUVEC (Fig. 1B). In fact, the exposure time was at least 30 times greater to detect a much lower level of MTf in HUVEC compared to HMEC-1 (Fig. 1B).

3.2. hr-sMTf stimulates plasminogen activation

Since hr-sMTf interacts with scu-PA and plasminogen [41], we characterized the effect of hr-sMTf on the in vitro plasminogen activation by u-PA (Fig. 2). Because u-PA represents the active form of the zymogen scu-PA, we observed a higher plasminolytic activity in the presence of

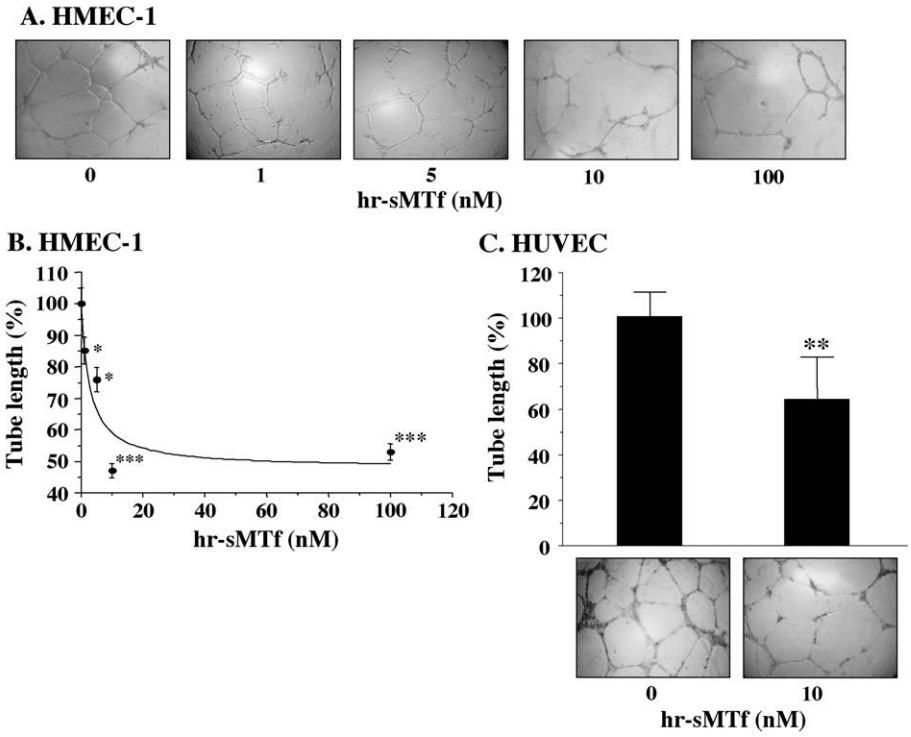

Fig. 4. Exogenous hr-sMTf inhibits the tubulogenesis of HMEC-1 and HUVEC. HMEC-1 were grown onto Matrigel-coated wells in the presence of increasing concentrations of hr-sMTf as described in Materials and methods. (A) Photos (original magnification, ×40) obtained from a representative experiment. (B) The results were expressed as the percentage of capillary-like structures of hr-sMTf-treated HMEC-1 compared to untreated cells. Data represent the means±SD of results obtained from three different experiments performed in triplicates. (C) HUVEC were grown onto Matrigel-coated wells in the presence or absence of hr-sMTf as described in Materials and methods. Statistically significant differences with control values are indicated by *P<0.05, **P<0.01 and ***P<0.001 (Student's t test). Photos (original magnification, ×40) obtained from a representative experiment are shown.

plasminogen with u-PA compared to scu-PA. When hr-sMTf is added to plasminogen, the VLK-pNA hydrolysis is four-fold higher for scu-PA and twofold higher for u-PA after 180 min. Control experiments performed with hr-sMTf alone demonstrate that sMTf itself does not generate plasmin when it is added to plasminogen. This also indicates that hr-sMTf is not contaminated by plasminogen activators. These results shows that hr-sMTf positively affects the in vitro activation of plasminogen by both scu-PA and u-PA in solution.

3.3. Requirement of hr-sMTf native conformation in the inhibition of HMEC-1 cell movement

Since hr-sMTf affects the in vitro plasminogen activation by scu-PA and u-PA, key players in cell movement, we further investigated the modulation of this process by hr-sMTf (Fig. 3). First, to determine whether HMEC-1 movement was plasmin dependent (Fig. 3A), we measured HMEC-1 cell movement in the presence of plasmin inhibitors. Both aprotinin (1 μM) and EACA (100 mM) inhibited by about 50% the HMEC-1 movement, indicating that plasmin is involved in HMEC-1 movement. Secondly, HMEC-1 cell movement was examined in the presence of exogenous hr-sMTf (Fig. 3B). hr-sMTf, at 10 and 100 nM, inhibits the movement of HMEC-1 by 34% and 50%, respectively. This inhibition is similar to the one observed in the presence of plasmin inhibitors. Moreover, the inhibition of HMEC-1 cell movement was completely lost when hr-sMTf was boiled for 30 min at 100 °C prior to the assay (Fig. 3C). This result demonstrates that a native conformation of hr-sMTf is required to inhibit HMEC-1 cell movement. Under these conditions, the adhesion of HMEC-1 on gelatin was found unaffected by hr-sMTf (data not shown), indicating that hr-sMTf inhibits cell movement without modulating cell adhesive properties.

3.4. Exogenous hr-sMTf inhibits EC tubulogenesis

In the present study, HMEC-1 and HUVEC cells growth on Matrigel generate a stabilized network of capillary-like structures. This is shown by the complexity of the tubular network per field in control cells (Fig 4). We therefore determined the effects of exogenous hr-sMTf on HMEC-1 (Fig. 4A–B) and HUVEC (Fig. 4C) tubulogenesis. The generation of capillary-like tubular structures was strongly reduced when hr-sMTf was added during the experiments. Indeed, hr-sMTf reduced the capillary-like tube formation of HMEC-1 in a dose-dependent manner and reached a maximal tubulogenesis inhibition at 10 nM (Fig. 4A–B). We also confirmed the hr-sMTf tubulogenesis inhibition on another EC type (HUVEC), using an intermediate hr-sMTf concentration (10 nM). At this hr-sMTf concentration, the capillary-like tube formation of HUVECs was reduced by 38% (Fig. 4C). These results show that low concentrations of hr-sMTf inhibit EC tubulogenesis.

3.5. Exogenous hr-sMTf down-regulates u-PAR and LRP protein expression

To identify a potential mechanism by which hr-sMTf inhibited in vitro EC movement and tubulogenesis, the effect of hr-sMTf on the protein expression of both u-PAR and LRP was measured by Western blotting and RT-PCR analysis (Fig. 5A–B). HMEC-1 cells were incubated for 18 h with or without hr-sMTf. Treatment with hr-sMTf significantly down-regulated u-PAR and LRP protein expression. In fact, exposure of HMEC-1 to hr-sMTf at 10 and 100 nM reduced u-PAR protein expression in cell

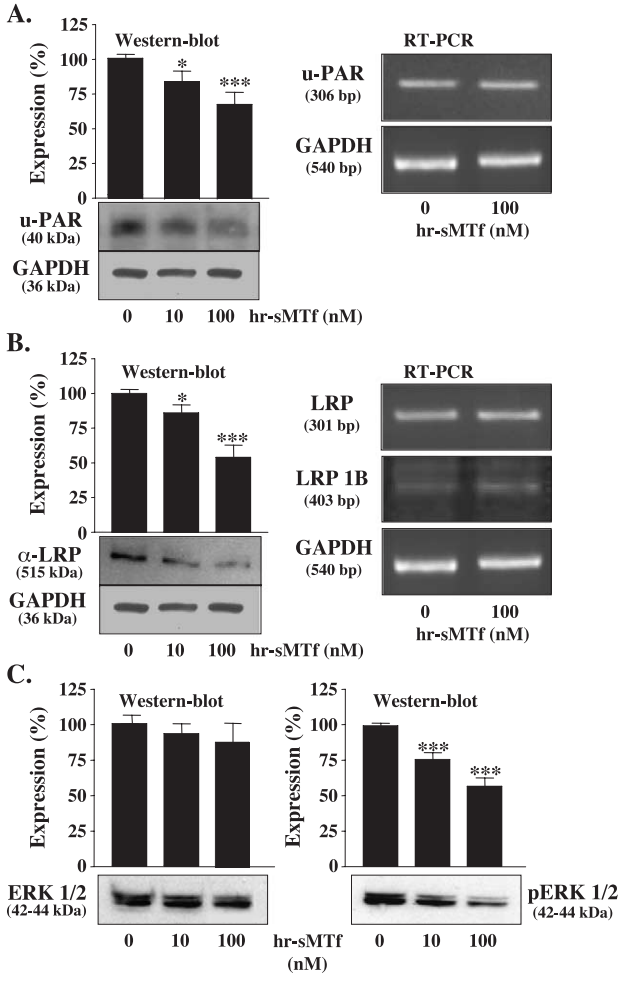

Fig. 5. Exogenous hr-sMTf down-regulates u-PAR and LRP protein expression. HMEC-1 were treated for 18 h with or without hr-sMTf. Following this treatment, proteins from cell lysates were resolved by SDS-PAGE, whereas total RNA was isolated and gene products were amplified by RT-PCR. Immunodetections and RT-PCR of u-PAR (A), LRP (B) as well as immunodetection of ERK 1/2 and pERK 1/2 (C) were performed as described in Materials and methods. Immunodetections obtained from a representative experiment are shown. Immunodetection results were expressed as a percentage of protein expression detected in hr-sMTf-treated cells compared to untreated cells. Data represent the means±SD of results obtained from three different experiments. For RT-PCR analysis, results obtained from a representative experiments are shown (n=3). Statistically significant differences with control values are indicated by *P<0.05 and ***P<0.001 (Student's t test).

lysates by 20% and 40%, respectively (Fig. 5A). The same concentrations decreased LRP protein expression by 20% and 50%, respectively (Fig. 5B). Since hr-sMTf modulated u-PAR and LRP protein expression, the mRNA expression of LDL-R family gene and u-PAR were estimated by RT-PCR in HMEC-1 treated or not with hr-sMTf (Fig. 5A–B). Primers used for this characterization are specific for each member of the LDL-R family since they only recognized their corresponding cDNA sequence in a nucleotide–nucleotide BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). Expression of the different members of the LDL-receptor family was first investigated in untreated HMEC-1 cells. Under the conditions used for RT-PCR analysis, LRP, LRP 1B, LDL-R and LRP 8 were clearly amplified whereas LRP 2 and LRP 5 products were almost undetectable (data not shown). Following hr-sMTf treatment, the mRNA levels of u-PAR, LRP and LRP 1B was unchanged in treated cells as compared to control cells (Fig. 5A–B). Also, the mRNA levels of LRP 2, LDL-R, LRP 8 was unchanged in treated cells as compared to control cells (data not shown). Since u-PAR and LRP gene expression were unaffected by hr-sMTf, these results indicate that hr-sMTf regulation of u-PAR and LRP expression must take place at the protein level.

Recently, it has been shown that a down-regulation of u-PAR expression inhibits ERK 1/2 signaling with concomitant suppression of colon cancer cell migration and invasion [42]. Thus, to determine if the hr-sMTf-mediated down-regulation of u-PAR correlates with ERK 1/2 signaling inhibition, we evaluated the effects of hr-sMTf on ERK 1/2 protein expression and phosphorylation levels by Western blot analysis (Fig. 5C). The ERK 1/2 level was unchanged following hr-sMTf treatment in HMEC-1 (Fig. 5C). In contrast, the pERK 1/2 level was significantly decreased by 25% and 40% in HMEC-1 treated with 10 and 100 nM hr-sMTf (Fig. 5C), respectively. These results show that hr-sMTf affects ERK 1/2 signaling pathway by down-regulating u-PAR protein expression and thus leading to a reduction of cell movement and tubulogenesis.

3.6. Exogenous hr-sMTf modulates u-PAR and LRP cell surface expression

In view of the fact that u-PAR and LRP protein expression is affected by exogenous hr-sMTf and that the amount of u-PAR and LRP at the membrane surface is a key element in plasmin formation and cell movement, we determined the u-PAR and LRP levels at the cell surface by FACS following hr-

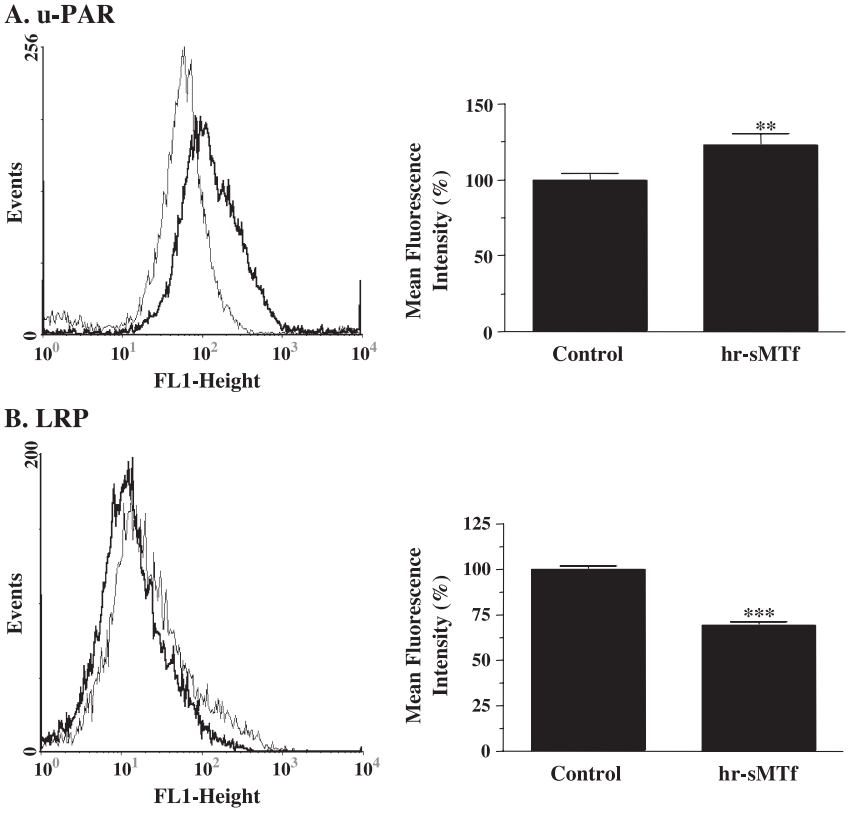

Fig. 6. Exogenous hr-sMTf modulates u-PAR and LRP cell surface expression. HMEC-1 were treated for 18 h with or without 100 nM of hr-sMTf. Flow cytometric analysis of cell surface u-PAR (A) and LRP (B) levels was performed as described in Materials and methods. Control (thin line) or treated HMEC-1 (bold line) were labeled with anti-u-PAR antibody or with anti-α-LRP antibody and detected with goat anti-mouse IgG-Alexa488. These results are representative of three different experiments. Results were corrected for the background fluorescence intensity measured with a nonspecific IgG1 and expressed as mean fluorescence intensities. Data represent the means±SD of three different experiments. Statistically significant differences are with control values indicated by $^{**}P<0.01$ and $^{***}P<0.001$ (Student's t test).

sMTf treatment (Fig. 6). HMEC-1 were incubated with or without hr-sMTf (100 nM) for 18 h, the most suitable time period for this assay, where hr-sMTf exerts its optimal inhibition of tubulogenesis. Cell surface u-PAR expression was assessed in control (thin line) and hr-sMTf-treated cells (bold line) by FACS (Fig. 6A). The mean fluorescence intensity associated with the detection of cell surface u-PAR is significantly higher by 25% following hr-sMTf treatment. Cell surface LRP expression was also assessed by FACS in control (thin line) and treated cells (bold line) (Fig. 6B). The mean fluorescence intensity associated with the detection of cell surface LRP is significantly decreased by 30% following hr-sMTf treatment. These results suggest that hr-sMTf treatment significantly increased u-PAR, while it concomitantly decreased LRP expression at the cell surface of HMEC-1.

3.7. Exogenous hr-sMTf decreases free and active u-PAR at the cell surface

We next measured the binding of ^{125}I-uPA•PAI-1 complexes on HMEC-1 to evaluate whether cell surface u-PAR was free or bound to u-PA or uPA•PAI-1 complex (Fig. 7A). As for the tubulogenesis assay, HMEC-1 were exposed to hr-sMTf (100 nM) for 18 h. Following this treatment, the binding of ^{125}I-uPA•PAI-1 complexes was measured at 4 °C in control and treated cells. The radioactivity associated with the binding of ^{125}I-uPA•PAI-1 complexes was reduced by about 23% following hr-sMTf treatment. Following this treatment, the plasmin activity at the cell surface of control and treated cells was also measured. When cells were treated with hr-sMTf, plasminogen activation was inhibited by 95% (Fig. 7B). Altogether, these results suggest that hr-sMTf treatment decreased the free and active u-PAR at the cell membrane of HMEC-1 leading to a reduction in the capacity of HMEC-1 to activate plasminogen. This mark reduction of plasminolytic capacity of HMEC-1 cells by hr-sMTf could explain the inhibition of HMEC-1 movement and tubulogenesis.

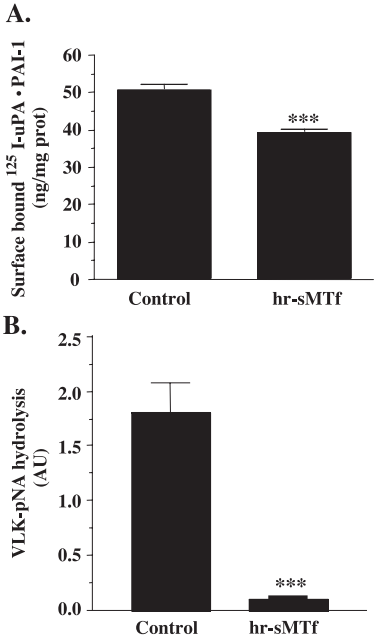

Fig. 7. Exogenous hr-sMTf decreases free and active u-PAR at the cell surface. HMEC-1 were treated for 18 h with or without 100 nM of hr-sMTf. (A) Binding of ^{125}I-uPA•PAI-1 complexes on HMEC-1 cell surface. Following cell treatment with hr-sMTf, binding of ^{125}I-uPA•PAI-1 complexes was performed as described in Materials and methods. Data represent the means±SD of three different experiments. (B) Effect of hr-sMTf on plasminolytic activity. Following cell treatment, the plasminolytic activity was measured in the presence of 30 nM plasminogen as described in Materials and methods. Statistically significant differences with control values are indicated by ***P<0.001 (Student's t test).

4. Discussion

MTf was originally discovered as a surface marker of malignant melanoma cells [1]. Since MTf is a homologue of transferrin, it has been postulated that MTf might be involved in iron transport. Subsequently, however, several studies concluded that membrane-bound MTf and sMTf, which exists at trace quantities in the serum [19], plays only a minor role in Fe uptake [12,13,15]. Thus, the functions of both MTf remained largely unexplored. However, we have recently shown that hr-sMTf could interact with plasminogen and scu-PA, two members of the u-PAR/LRP plasminolytic system [41]. In the present study, the in vitro enzymatic assay for plasmin indicates that hr-sMTf also increases the plasminogen activation by u-PA. These results suggest that hr-sMTf can affect molecular processes involving scu-PA as well as u-PA-dependent plasminogen activation.

Here, we demonstrated by using SDS-PAGE that hr-sMTf migrates at 60 and 73 kDa under nonreducing conditions and as a single band at 97 kDa under reducing conditions. These results on this 97 kDa monomeric sialoglycoprotein are similar to those previously reported by Desrosiers et al. [19]. In addition, using Western blotting analysis, we found that L235 antibody immunodetect hr-sMTf as well as endogenous MTf in HMEC-1 cells. MTf expressed in HMEC-1 migrates at a higher molecular weight compared to recombinant sMTf. This is in agreement with the 27 C-terminal amino acids deletion used for the production of hr-sMTf. Because hr-sMTf is produced in baby hamster kidney cells, the electrophoretic migration can also reflect a different glycosylation pattern.

The plasminogen/plasmin system plays a crucial role not only in blood clot lysis but also in various physiological and pathological events including inflammation, tissue remodeling, tumor metastasis and angiogenesis, where localized proteolysis is required. Thus, because of the importance of the plasminolytic system in EC movement and tubulogenesis [43], the impact of hr-sMTf on EC plasminolytic activity, movement and tubulogenesis has been investigated. Paradoxically to the in vitro results, the basal capacity of HMEC-1 cells to activate plasminogen surprisingly

decreased following hr-sMTf treatment. This, in part, can explain the dose-dependent inhibition of EC movement and tubulogenesis by hr-sMTf. Since several mechanisms can be involved in the inhibition of EC tubulogenesis, a complete inhibition may be difficult to reach. For example, a specific inhibitor of VEGF receptor inhibits EC tubulogenesis in a dose-dependent manner and reach a maximum inhibition of 45% at 10 μM [44]. This concentration is far higher than that of hr-sMTf (10 nM), indicating that low concentrations of hr-sMTf effectively inhibit tubulogenesis. Moreover, these low values are compatible to previously reported effective concentrations for angiostatins including K1–3, K1–4 and K1–4+7 residues of K5, which exert anti-endothelial activity at 10–300 nM [20,45,46].

Several reports showed that the u-PAR and LRP receptors play a key role in signal transduction as well as in the regulation of melanoma cell migration and angiogenesis [47–50]. One possible explanation for the hr-sMTf-mediated inhibition of the basal capacity of HMEC-1 cells to activate plasminogen, could be related to the decreased expression of u-PAR and LRP as well as to the decrease in free and active u-PAR at the cell surface (Fig. 8). Moreover, an increase in LRP expression seems to correlate with both the expression of u-PAR and the malignancy of the astrocytoma, suggesting that LRP plays a role in facilitating the invasiveness of glioblastomas by regulating cell surface proteolytic activity [51]. LRP-deficient cells also demonstrated increased levels of cell-surface u-PAR [52]. Recently, it was proposed that a negative regulation of the regeneration of u-PAR at the cell surface was correlated with a diminished rate of cell migration [53]. Also, using an in vitro model of the blood–brain barrier, we showed that hr-sMTf transcytosis might involved LRP [54]. This result indicates that LRP is a potential receptor for hr-sMTf transport. In light of this, our data strongly suggest that hr-sMTf treatment reduces the free and active u-PAR regeneration at the cell surface, by both increasing the internalization of his scavenger receptor (LRP) and reducing the LRP protein expression. Because of the importance of plasminogen activation at the cell surface, which leads to plasmin generation and promotes angiogenesis, the present study provides new evidences that exogenous hr-sMTf affects receptors involved in u-PA and t-PA-dependent plasminogen activation. This modulation leads to the inhibition of the plasminogen activation at the cellular surface as well as to the inhibition of EC movement and tubulogenesis. Nevertheless, we can expect that hr-sMTf could also modulate other protein(s) involved in plasminogen activation and tubulogenesis.

We also report that ERK 1/2 activation to pERK 1/2 are reduced following hr-sMTf treatment in HMEC-1. The importance of ERK 1/2 in regulating cellular migration after the down-regulation of uPAR has been previously demon-

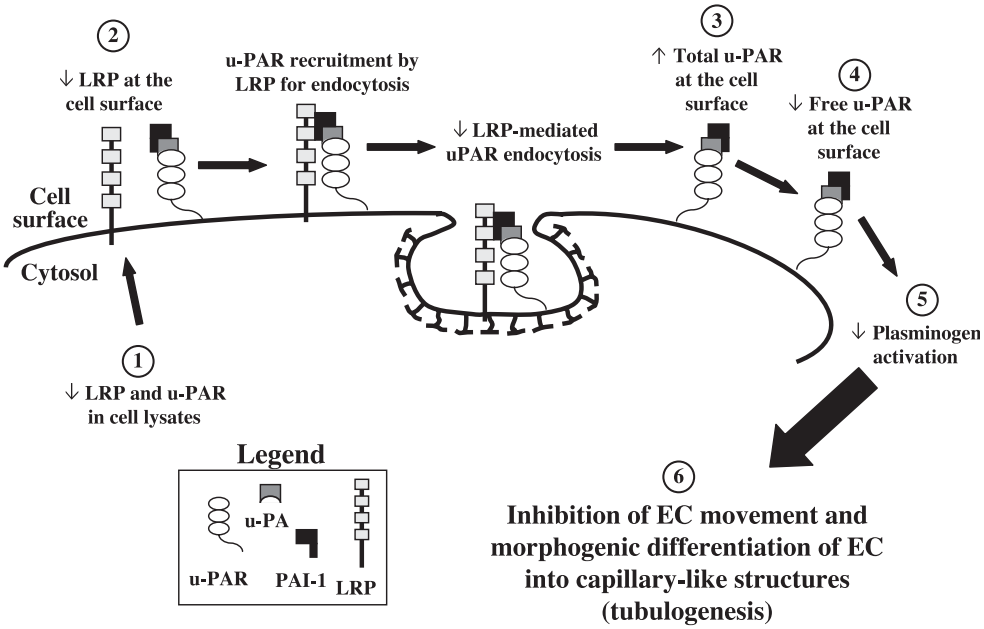

Fig. 8. Schematic representation of the hr-sMTf treatment effects on the u-PAR/LRP plasminolytic system. This schematic representation summarizes how hr-sMTf affects the turnover of the free and active u-PAR at the HMEC-1 cell surface. In normal condition, u-PA bound to u-PAR catalyzes the conversion of plasminogen to plasmin, which in turn degrades the extracellular matrix and facilitates cell migration and tubulogenesis [30]. The binding of PAI-1 to u-PA inactivates u-PA, triggers the recruitment of u-PAR by LRP and the formation of a quaternary complex [uPAR•uPA•PAI-1•LRP] for endocytosis. Then, LRP and u-PAR are sorted into recycling vesicle, whereas u-PA•PAI-1 complexes are degraded. This recycling permits the regeneration of free and active u-PAR at the plasma membrane. In the present study, hr-sMTf treatment decreases the u-PAR and LRP expression in cell lysates ①. hr-sMTf also decreases LRP at the cell surface ② and increases total u-PAR at the cell surface ③, suggesting that recombinant sMTf reduces the LRP-mediated u-PAR endocytosis. The lower capacity to bind the u-PA•PAI-1 complex also indicates that hr-sMTf decreases the free and active u-PAR at the cell surface ④. This is consistent with the mark decreased basal capacity of HMEC-1 to activate plasminogen ⑤. Overall, the effects of hr-sMTf treatment on the u-PAR/LRP plasminolytic system lead to the inhibition of EC movement and morphogenic differentiation into capillary-like structures (tubulogenesis) ⑥.

strated. Activated ERK 1/2 phosphorylates and thereby activates myosin light chain kinase, increasing cytoskeletal contractility that may promote cellular migration [55,56]. Activated ERK 1/2 also regulates integrin function, possibly deactivating certain integrins and promoting the disassembly of focal adhesions [57,58]. The regulation of ERK 1/2 activity may have profound effects on various physiologic processes that involve cellular movement. Thus, the down-regulation of ERK 1/2 phosphorylation following hr-sMTf treatment is in agreement with the down-regulation of u-PAR protein expression and the inhibition of movement and tubulogenesis by hr-sMTf.

In conclusion, we showed that hr-sMTf inhibits HMEC-1 cell movement as well as HMEC-1 and HUVEC tubulogenesis by affecting the u-PAR/LRP plasminolytic system. In fact, hr-sMTf disturbs the regeneration of the free and active u-PAR at the cellular surface by LRP and reduces the HMEC-1 capacity to generate plasmin from plasminogen. Consequently, these are the first evidences suggesting that hr-sMTf affects the EC movement and morphogenic differentiation into capillary-like structures. These results are also the first to suggest that the modulation of sMTf serum concentrations could be considered as a possible therapeutic approach in angiogenesis-related pathologies.

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