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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.