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Biochimica et Biophysica Acta 1763 (2006) 1024–1030
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.elsevier.com/locate/bbamcr

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

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Received 26 February 2006; received in revised form 8 August 2006; accepted 9 August 2006
Available online 11 August 2006

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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doi:10.1016/j.bbamcr.2006.08.006

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*