Thrombosis Research 132 (2013) 100–105

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

TM5275 prolongs secreted tissue plasminogen activator retention and enhances fibrinolysis on vascular endothelial cells

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ARTICLE INFO

Article history:
Received 10 January 2013
Received in revised form 5 March 2013
Accepted 1 April 2013
Available online 20 April 2013

Keywords:
fibrinolysis
plasminogen activator inhibitor-1 (PAI-1)
plasminogen activator inhibitor-1 (PAI-1) inhibitor
TM5275
total internal reflection fluorescence (TIRF) microscopy

ABSTRACT

Introduction: Elevated plasminogen activator inhibitor-1 (PAI-1) reduces fibrinolytic potential in plasma, contributing to thrombotic disease. Thus, inhibiting PAI-1 activity is clinically desirable. We recently demonstrated that tissue plasminogen activator (tPA) remains on the surface of vascular endothelial cells (VECs) after secretion in a heavy-chain dependent manner, which is essential for high fibrinolytic activity on the surface of VECs, and that PAI-1 dissociates retained tPA from the cell surface as a result of high-molecular weight complex formation. Based on the model whereby amounts of tPA and its equilibrium with PAI-1 dynamically change after exocytosis, we examined how TM5275, a newly synthesized small molecule PAI-1 inhibitor, modulated tPA retention and VEC surface-derived fibrinolytic activity using microscopic techniques.
Materials and methods: The effects of TM5275 on the kinetics of the secretion and retention of green fluorescent protein (GFP)-tagged tPA (tPA-GFP) on VECs were analyzed using total internal reflection fluorescence microscopy. The effects of TM5275 on the generation of plasmin activity were evaluated by both plasminogen accumulation and fibrin clot lysis on tPA-GFP-expressing VECs using confocal laser scanning microscopy.
Results: TM5275 at concentrations of 20 and 100 μM significantly prolonged the retention of tPA-GFP on VECs by inhibiting tPA-GFP-PAI-1 high-molecular-weight complex formation. TM5275 enhanced the time-dependent accumulation of plasminogen as well as the dissolution of fibrin clots on and around the tPA-GFP-expressing cells.
Conclusions: The profibrinolytic effects of TM5275 were clearly demonstrated by the prolongation of tPA retention and enhancement of plasmin generation on the VEC surface as a result of PAI-1 inhibition.

Introduction

Tissue plasminogen activator (tPA) is a 68-kDa serine protease that initiates fibrinolysis by cleaving a single peptide bond in plasminogen to generate plasmin, which subsequently dissolves fibrin clots in the vasculature. Plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor superfamily, regulates both free tPA concentration and tPA activity in plasma by forming a high-molecular-weight complex with tPA [1–5]. Elevated levels of PAI-1 antigen and activity, which are frequently seen in dyslipidemia and metabolic syndrome, naturally reduce fibrinolytic potential in plasma, and contribute to the development of a variety of thrombotic diseases, including deep vein thrombosis, disseminated intravascular coagulation, and coronary artery diseases [6]. The inhibition of PAI-1 activity is thus expected to yield important cardio- and vascular-protective benefits [7], and much attention has been paid to developing inhibitors of PAI-1 [8–10]. One such inhibitor, TM5275, was newly identified by an extensive study of structure-activity relationships based on a lead compound (TM5007) obtained through virtual screening and docking simulations [11,12].

Recently, we successfully visualized the secretory dynamics of green fluorescent protein (GFP)-tagged tPA (tPA-GFP) expressed in vascular endothelial cells (VECs) using total internal reflection fluorescence (TIRF) microscopy, and demonstrated that tPA has unique, slow secretory dynamics and stays on VECs for long periods of time after opening of the secretory granules [13]. This sustained retention of tPA appeared essential for the effective expression of cell surface-associated plasmin activity and the associated fibrinolysis on VECs [14]. We also demonstrated that PAI-1 facilitates the dissociation of tPA from the surface of VECs by forming a high-molecular-weight complex. Thus, PAI-1 appeared to suppress tPA activity not only in plasma but also on the

Abbreviations: tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1; VEC, vascular endothelial cell; TIRF, total internal reflection fluorescence; HBS, HEPES-buffered solution; CLSM, confocal laser scanning microscope.

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http://dx.doi.org/10.1016/j.thromres.2013.04.003

surface of VECs. Based on these findings, we hypothesized that TM5275 might critically modulate PAI-1 activity on the VEC surface, where active tPA is constantly exocytosed and its equilibrium with PAI-1 changes dynamically, and thereby potentiate fibrinolytic activity triggered by VEC surface-retained tPA. To test this, we used microscopy techniques to explore the effects of TM5275 on both tPA retention and the expression of fibrinolytic activity on VECs.

Materials and Methods

Cell Culture and Transfection

The human umbilical vein endothelial cell-derived VEC line EA.hy926, which retains endothelial cell-specific functions [15], including fibrinolytic characteristics [16], was kindly provided by Dr C.J. Edgell. We cultured the cells in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO) supplemented with 4.5 g/L glucose and 10% fetal bovine serum at 37 °C under 5% CO_2 in a humidified atmosphere. The cells were cultivated on 35-mm glass bottom dishes (Asahi Techno Glass, Tokyo, Japan) for fluorescence imaging, and on 6-well plates for supernatant analyses. Construction of the tPA-GFP plasmid was described previously [13]. The cells were transfected with the plasmid by lipofection using TransIT-LT1 (Mirus, Madison, WI) at 100% confluence. Experiments were performed within 1 day after transient transfection.

Solutions and Materials

A HEPES-buffered solution (HBS) composed of 140 mM NaCl, 5 mM KCL, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES-NaOH (pH 7.3) supplemented with 3% bovine serum albumin (BSA) (HBS/3%BSA) was used as recording medium, and cells were kept in this solution at 37 °C on the microscope stage (INUG2-ONID-BE; Tokai Hit, Shizuoka, Japan) in all imaging studies. Recombinant tPA (rtPA) was kindly provided by Toyobo-Daiichi (Osaka, Japan), and recombinant PAI-1 (rPAI-1; active, nonglycosylated form) was purchased from Oxford Biomedical Research (Rochester Hills, MI). The ELISA kit for PAI-1 antigen was purchased from Hyphen BioMed (Neuville-sur-Oise, France). Human Glu-plasminogen, purified from fresh-frozen human plasma, and human fibrinogen (Enzyme Research Laboratories, IN) were labeled with Alexa Fluor 568 (Invitrogen, OR) (plg-568) and Alexa Fluor 647 (fbg-647), respectively. Human thrombin was purchased from Benesis (Osaka, Japan). PAI-1 inhibitors, TM5275 [12], synthesized by Hamari Chemicals Ltd (Osaka, Japan), and Tiplaxtinin (Axon Medchem BV, Netherlands) were dissolved in DMSO at a concentration of 0.1% for all studies.

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The effects of TM5275 on the formation of a tPA-PAI-1 complex were evaluated in a purified system using SDS-PAGE. After incubation with TM5275 at concentrations of 0 (solvent alone), 20, and 100 μM in HBS for 10 min at 37 °C, rPAI-1 (final concentration, 250 nM) was incubated with rtPA (final concentration, 270 nM) for 30 min at 37 °C. After mixing with sample buffer (non-reducing), the mixture was subjected to 10% SDS-PAGE, and the protein bands were stained with Coomassie Brilliant Blue.

Fibrin Autography

To evaluate the effects of TM5275 on the ability of PAI-1 to form high-molecular-weight complexes with tPA either on cultured EA.hy926 cells or in the supernatant, the amounts of tPA-PAI-1 complex and free tPA were semi-quantitated by fibrin autography. Culture media from tPA-GFP-expressing or non-expressing EA.hy926 cells were collected after 3 h of incubation in the presence of 0 (solvent alone), 20, and 100 μM TM5275 at 37 °C, centrifuged at 3,000 ×*g* for 10 min to remove cell debris, mixed with SDS sample buffer, and subjected to 10% SDS-PAGE. tPA-dependent activities were then detected by plasminogen-rich fibrin indicator gels after separation of the protein bands as previously reported [17].

TIRF Microscopic Analysis

We employed a TIRF microscopy unit to evaluate the time that tPA-GFP was retained (tPA-GFP retention time) on cell membrane surfaces after exocytosis from its secretory granules in EA.hy926 cells. This unit enabled us to detect only those fluorophores existing near the plasma membrane facing towards the glass bottom side, as previously described [13]. Briefly, cells were imaged with an inverted microscope (IX81, Olympus, Tokyo, Japan) equipped with a 60X/1.45 numeric aperture oil-immersion objective (for TIRFM, Olympus) to generate the so-called "evanescent field," which illuminates to a depth of ≤100 nm from the glass coverslip into the specimen. We introduced a neutral density filter (Edmund Optics, NJ) to reduce laser intensity to 6%, by which bleaching of intracellular fluorophores was suppressed to less than 10%. GFP was excited at 488 nm, and the emitted light was collected through a 510/23-nm bandpass filter with a 506-nm dichroic mirror. The fluorescence images were captured and recorded every 500 ms through a cooled (−65 °C) high-sensitivity electron multiplier charge-coupled device (EM-CCD; ImagEM, Hamamatsu Photonics, Hamamatsu, Japan) camera controlled by HC Image (Hamamatsu Photonics). We measured the fluorescence intensity of the GFP in each tPA-GFP secretory granule that was exocytosed using the AQUACOSMOS Imaging Station (Hamamatsu Photonics). The "tPA-GFP retention time" $T_{F1/2}$ was defined as the time required for the fluorescence intensity at a single granular spot to decline to one-half of its peak value during the releasing process of tPA-GFP.

Plasminogen-accumulation Analysis

After treatment with 100 μM TM5275 or solvent in HBS/3% BSA for 30 min at 37 °C, tPA-GFP-expressing cells were incubated with human plasminogen (0.5 μM) containing plg-568 (20 nM). The accumulation of plg-568 on or around the cell surface was then analyzed every 10 min with a confocal laser scanning microscope (CLSM; FV1000, Olympus) equipped with a 60X oil-immersion objective lens that captured the fluorescence of plg-568 at wavelengths from 570 nm to 670 nm. Just before the end of each experiment, we added 2.5 μg/mL Cell Mask Deep Red plasma membrane stain (PM; Molecular Probes, Invitrogen, OR) to identify the localization of tPA-GFP-expressing cells. We created a region of interest (ROI) around a single cell, including the pericellular area at the most basal focal plane, and measured the fluorescence intensity using FV10-ASW software (Olympus). Because the mean fluorescence intensity within the ROI increased linearly for 10 min, we calculated the slope of the fluorescence increase over time, representing a time-dependent accumulation of plg-568, and referred to this as dF-plg.

Fibrin Clot Lysis Imaging

tPA-GFP-transfected cells were preincubated with 100 μM TM5275 or solvent in HBS/3%BSA for 30 min at 37 °C. Fibrin clots were then formed over the cells by mixing 0.5 μM human plasminogen, containing 20 nM plg-568, 2 U/mL thrombin, and 1 mg/mL human fibrinogen containing 10 μg/mL fbg-647, in HBS/3%BSA. After fibrin clots were formed on the VECs, we started to collect images every 10 min through an automatically selected dichroic mirror and an appropriate range of