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Tissue plasminogen activator enhances mobilization of endothelial progenitor cells and angiogenesis in murine limb ischemia☆,☆☆

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ABSTRACT

Background: It has been reported that plasmin induces migration of endothelial cells. We hypothesized that tissue plasminogen activator (tPA) enhanced endothelial progenitor cell (EPC) mobilization from bone marrow (BM) into the circulation and angiogenesis in murine critical limb ischemia (CLI).
Methods: Forty male B6 mice were randomly divided into group 1 (control), group 2 [control + recombinant tPA (intra-venous 4 mg/kg)], group 3 (CLI) and group 4 (CLI + tPA).
Results: The results demonstrated higher MMP-9 activity in BM and circulatory SDF-1α level in groups 2 and 3 than in group 1, and highest in group 4 (all $p<0.01$) at 18 h after CLI. Compared with circulation, SDF-1α level in BM showed an opposite trend (all $p<0.03$). The circulating EPC (C-kit/CD31, Sca-1/KDR, CXCR4/CD34) levels at 18 h after CLI were higher in group 2 than in groups 1 and 3, and highest in group 4 (all $p<0.03$). EPC (C-kit/CD31, Sca-1/KDR) levels in BM were lower in group 1 than in groups 2 to 4 (all $p<0.03$), whereas number of CXCR4/CD34-positive EPCs in BM did not differ among the four groups at 18 h after CLI. Protein expressions of SDF-1α, CXCR4, eNOS, and VEGF, numbers of CD31+, CXCR4+, SDF-1α+, and vWF+ cells through immunofluorescent staining, numbers of small vessels (<15 μm) and blood flow measured by Laser Doppler in an ischemic area were significantly higher in group 4 than in group 3 (all $p<0.005$) at day 14 after CLI.
Conclusion: tPA treatment enhanced number of circulating EPCs, angiogenesis, and blood flow to ischemic tissue in a murine model of limb ischemia.

1. Introduction

Although thrombolytic therapy using tissue plasminogen activator (tPA) remains one of the gold standards in the treatment of acute ST-segment elevation myocardial infarction (STEMI) [1,2], the possible mechanisms underlying the beneficial effects of tPA therapy in improving left ventricular function other than restoration of blood flow of the infarct-related artery (IRA) have not been investigated.

Previous studies have revealed the essential role of proteolytic enzymes in invasion and migration of cancer cells through cleavage of extra-cellular matrix (ECM) [3,4]. Similarly, mobilization of endothelial progenitor cells (EPCs) from the quiescent niche of bone marrow (BM) to the circulation has also been reported to be related to the activation of proteolytic enzymes, including elastase, cathepsin G, and matrix metalloproteinases (MMPs) [5,6]. It has been demonstrated that BM stromal cells express membrane-bound C-kit ligand (C-kit-L) which binds to the membrane receptor C-kit of EPCs when the ligand is in its soluble form [4–6]. These proteolytic enzymes have been reported to cleave the ECM- or cell membrane-bound molecules responsible for the bond between EPCs and BM stromal cells [4–6]. For instance, it has been shown that MMP-9 acts by cleaving the membrane-bound C-kit-L to its soluble form which then interacts with the EPC C-kit receptor to elicit the signal crucial for BM-EPC differentiation and mobilization to the systemic circulation [4–7].

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A higher circulatory stromal cell-derived factor (SDF)-1α concentration compared with that in BM creates a concentration gradient that has been demonstrated to play a crucial role in modulating EPC mobilization from BM into the circulation [7–9]. SDF-1α binds specifically to the receptor CXCR4 [8,10–12] expressed on the surface of EPCs, especially CD34+ cells [6,9,13]. Therefore, SDF-1 acts as a principal chemokine that promotes the mobilization of EPCs from BM after MMP-9-mediated cleavage of membrane-bound C-kit-L [6,14,15]. Furthermore, SDF-1α, which is expressed in activated platelets, smooth muscle cells, and ischemic cell/tissue, has been shown to be markedly increased [16,17] in response to ischemic stimulus and mediates the recruitment of progenitor cells along the hypoxic gradients towards the ischemic zone [13,15,18].

On the other hand, tPA, which cleaves plasminogen (inactive form) to plasmin (active form), has been shown to be involved in the proteolytic degradation of ECM components and enhance MMP-9 activity [3,19]. Indeed, plasmin has been reported to induce migration of endothelial cells [18]. Accordingly, this study investigated whether tPA therapy could improve blood flow to ischemic tissue through enhancement of the mobilization and migration of EPCs for angiogenesis in the setting of murine critical limb ischemia (CLI).

2. Methods

2.1. Ethics

All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at our institute and performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, National Academy Press, Washington, DC, USA, revised 1996). The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.

2.2. Animal model of critical limb ischemia

Twelve-week-old male B6 mice (n = 40), weighing 22–25 g, (Charles River Technology, BioLASCO Taiwan Co., Ltd., Taiwan), were equally divided (i.e. n = 10/each group) into group 1 (normal control), group 2 [normal control + tPA (intra-venous 4 mg/kg)], group 3 [critical limb ischemia (CLI) only], and group 4 (CLI + tPA). tPA was given at 3 h after induction of CLI.

Mice in groups 3 and 4 were anesthetized with inhalational isoflurane. The mice were placed in a supine position on a warming pad at 37 °C with the left hind limbs shaved. Under sterile conditions, the left femoral artery, small arterioles and circumferential femoral artery were exposed and ligated over their proximal and distal portions before being excised. To avoid the presence of collateral circulation, the branches were removed together. However, the veins were left intact during the procedure. After the procedure, the wound was closed and the animal was allowed to recover from anesthesia in a portable animal intensive care unit (ThermoCare®) for 24 h.

2.3. The rationale of tPA dosage in the study

Six mice were equally divided into three groups each of which received one testing dose of tPA. Totally three different doses of tPA (2.0 mg/kg, 4.0 mg/kg, and 6.0 mg/kg) were utilized in the current study to test its dose-dependent effects on EPC mobilization. Preliminary results from flow cytometry identified a tPA dosage of 6.0 mg/kg as most effective in increasing EPC numbers in the circulation at 18 h after the CLI procedure. However, postoperative bleeding complication was noted in the animals after such a high dosage. Further tests on the two other dosages of tPA demonstrated a superiority of a tPA dose of 4.0 mg/kg to 2.0 mg/kg in EPC mobilization into the circulation. Besides, no bleeding complication was noted after a tPA dosage of 4.0 mg/kg which was, therefore, adopted in this study. The protocol was designed to give only one dose of tPA to the animals to mimic the clinical scenario in which patients with ST elevation myocardial infarction received only a single therapeutic dose of tPA.

2.4. Measurement of blood flow with Laser Doppler flowmetry

Mice were anesthetized by inhalational isoflurane prior to CLI induction and on days 2 and 14 after CLI procedure prior to being sacrificed. The procedure and protocol were based on our recent report [20]. Briefly, the mice were placed in a supine position on a warming pad at 37 °C and the blood flow was detected in both inguinal areas by a Laser Doppler scanner (moorLDLS, Moor, Co., UK). The normal and ischemic regions were first identified for measurement of the blood flow. The machine then focused on the regions and automatically scanned the intensity of blood flow (i.e., exhibited as red, green or mixed color). The computer of the Laser Doppler scanner finally automatically read and calculated the intensity of the blood flow. All data were collected and put into a computer for further analysis. The ratio of flow in left (ischemic) leg and right (normal) leg was computed. The rats were sacrificed and the bicep muscle was collected for individual study.

2.5. Determination of SDF-1α level in bone marrow and circulation and MMP-9 activity in bone marrow

To determine SDF-1α levels in BM and circulation and MMP-9 activity in BM at 18 h after CLI procedure with tPA treatment, another 24 mice (i.e. 6 in each group) were utilized for this study. These mice were sacrificed at 18 h after the procedure and the plasma from both BM and circulation was collected for determining SDF-1α level using ELISA analysis and the supernatant from BM was collected for measuring MMP-9 activity using zymography analysis according to our recent report [21].

The blood samples were stored at −80 °C until SDF-1α analysis which was performed in batches at the end of the experiment. Serum SDF-1α concentration was assessed by duplicated determination with a commercially available ELISA kit (B & D Systems, Inc. Minneapolis, MN). The lower detection limit was 5 pg/mL. Intra-individual variability in SDF-1α level was assessed in each group. The mean intra-assay coefficients of variance were all less than 4.0%.

2.6. Construction of SDF-1α over-expression vector

The full-length cDNA of SDF-1α was synthesized and amplified with primer set 5′-atgaacgccaaggtcgtggtc-3′ and 5′-cttgtttaaagctttctccag-3′. Total RNA extracted from human umbilical vein endothelial cell (HUVEC) was used as template, while RT-PCR was performed with MMLV (Promega) and platinum Taq (Invitrogen). Synthesized cDNA was then cloned to pDsredMonomer-N1 (Invitrogen) with cloning sites Bgl II and Xho I. The constructed SDF-1α over-expression vector was further sequenced for examining sequences.

2.7. Determine the effect of tPA treatment on SDF-1α protein expression and MMP-9 activity in HUVECs

To determine whether tPA therapy would enhance SDF-1α and reduce MMP-9 protein expressions in HUVECs, stepwise increased concentrations of tPA (0, 10, 50 and 250 nM) were used in HUVEC cultures. The HUVECs (1.0×10^5 cells) were co-cultured with different concentrations of tPA for 24 h in M199 culture medium with n = 6 for each in vitro study. The cultured HUVECs were then collected for Western blot analysis.

2.8. Transwell migratory assay for human umbilical vein endothelial cell (HUVEC)

To determine the impact of tPA and SDF-1α on regulating cellular migratory ability, the transwell system and human umbilical vein endothelial cell (HUVEC) were used (Fig. 2A). Transwell membranes (5 μm; Costar, Germany) were coated on both sides with fibronectin (2.5 μg/mL; Roche, Mannheim, Germany) overnight at 4 °C. For the lower chamber, HUVECs (5×10^4 cells/well) were cultured on 24-well plates and then either treated with tPA (0–250 nM) or transfected with SDF-1α over-expression vector (0–2 μg/well) for 12 h. After incubation, supernatants were removed and M199 medium (Gibco, Carlsbad, CA, USA, USA) with 10% FBS (Gibco, Carlsbad, CA, USA, USA) were added. In the meanwhile, 5×10^4 HUVECs were resuspended in M199 medium with 0.5% FBS and loaded into the upper chamber. The assembled transwell system was then incubated at 37 °C in 5% CO_2 for 18 h. After incubation, cells that were remaining on the upper surface of the transwell membranes were mechanically removed and cells that had migrated to the lower surface were fixed with 4% formaldehyde. For quantification of cell nuclei, the migrated cells were stained with DAPI. Cells migrating into the lower chamber were counted in 5 random microscopic fields using a fluorescence microscope (Olympus, Tokyo, Japan) with software Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA).

2.9. Flow cytometric quantification of endothelial progenitor cells

For blood sampling at different time points (at 18 h and on day 14 after induction of CLI), cardiac puncture instead of the venous route was adopted for blood sampling using 30# needle. After treatment with red blood cell-lysing buffer, the cells remained were labeled with appropriate antibodies. Flow cytometric analysis for identification of cell surface markers was performed based on our recent reports [22]. Briefly, the cells were incubated for 30 min with primary antibodies, including PE-conjugated antibodies (against CD34, Sca-1, CD31, BD Biosciences), FITC-conjugated antibody against c-Kit (BD Biosciences), anti-CXCR4 (Abcam) and anti-KDR (NeoMarkers) antibodies which were further recognized by Alexa flour 488-conjugated secondary antibodies (Invitrogen). Isotype-identical antibodies (IgG) served as controls. Flow cytometric analyses were performed by utilizing a fluorescence-activated cell sorter (Beckman Coulter FC500 flow cytometer).

2.10. Western blot for protein expression

Equal amounts (10–30 μg) of protein extracts from ischemic quadriceps of the animals (n = 6 for each group) were loaded and separated by SDS-PAGE using 7% or 12% acrylamide gradients. The membranes were incubated with monoclonal antibodies