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Original article

Lesion-targeted thrombopoietin potentiates vasculogenesis by enhancing motility and enlivenment of transplanted endothelial progenitor cells via activation of Akt/mTOR/p70S6kinase signaling pathway

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ABSTRACT

Thrombopoietin (TPO), a physiological regulator of megakaryocyte and platelet development, is a multifunctional positive regulator in early hematopoiesis by hematopoietic stem cells. In this study, we investigated the effect of TPO on endothelial progenitor cells (EPCs) for therapeutic vasculogenesis *in vitro* and *in vivo*, and the intracellular signaling mechanism exerting the activity of EPCs. 7-day culture-expanded EPCs derived from human peripheral blood mononuclear cells were applied to each assay. Flow cytometry demonstrated the expression of c-Mpl, the receptor of TPO, in cultured EPCs. *In vitro* experiments revealed enhanced migration and survival of cultured EPCs by TPO. *In vivo*, TPO was intramuscularly administered into the foci of ischemic hindlimbs in athymic nude mice, immediately followed by intravenous injection of cultured EPCs, to assess the booster effect of TPO on vascular regeneration. At day 4 post-transplantation, transplanted EPCs were 1.7-fold higher in TPO-treated animals compared to control. At day 28, blood perfusion was recovered in the TPO-treated group, accompanied by an increase in microvascular density. The signaling transduction pathway underlying TPO-mediated activities of cultured EPCs was assessed by Western blotting. TPO induced sequential phosphorylations of Akt to p70S6kinase through mTOR. Inhibition of the PI3-kinase/Akt/mTOR/p70S6kinase signaling pathway negated the biological functions of cultured EPCs, either migration (by LY294002 for PI3-kinase and Rapamycin for mTOR) or survival and tubulogenesis (by Rapamycin). These findings provide evidence that TPO possesses booster potential for therapeutic vasculogenesis, by activating the PI3-kinase/Akt/mTOR/p70S6kinase pathway crucial to the biological activities of EPCs.

1. Introduction

Postnatal vasculogenesis, contributed by bone marrow (BM) derived endothelial progenitor cells (EPCs), has been recognized as one of the key events in physiological or pathological neovessel formation, concurrent with angiogenesis by resident endothelial cells (ECs) [1]. Based on a new dogma of blood vessel formation in postnatal life, "therapeutic vasculogenesis" targeting ischemic diseases has been recently developed, consisting of transplantation of total mononuclear cells from bone marrow, cultured EPCs, or autologous EPC fractioned in G-CSF-mobilized CD34+ cells [2,3]. However, the scarcity of original EPCs and the impairment of biological functions in patients suffering from risk factors of hyperlipidemia, hypertension, cigarette smoking, diabetes, aging, etc. [4] limits the efficacy of EPC transplantation on vasculogenesis in ischemic diseases. Therefore, methods of improving the quality and quantity of EPCs are desired for therapeutic purposes. Recently, several hematological regulators such as erythropoietin [5], stromal cell derived factor-1 (SDF-1) [6], granulocyte-colony stimulating factor (G-CSF), and estrogen have been described as versatile agents affecting the biological activities of endothelial lineage cells, including EPCs.

Thrombopoietin (TPO) is a hepatocyte-derived growth factor consisting of a 332 amino acid residue polypeptide which serves as a ligand for the TPO receptor (TPO-R; c-Mpl; CD110), which stimulates the development of human megakaryocyte (HuMK) progenitors and increases circulating platelets. Furthermore, TPO has recently been shown to play a pivotal role in hematopoietic stem/progenitor cells as well. Murine and human hematopoietic stem/progenitor cells have been shown to highly express c-Mpl [7], in which TPO stimulates VEGF expression in an autocrine manner to promote self-

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renewability [8]. Also, various studies have already revealed the ability of TPO to enhance the survival of hematopoietic stem/progenitor cells [9]. On the other hand, patients with unstable angina show higher serum levels of TPO as compared to healthy subjects or patients with stable angina [10], along with other heamatopoietic and angiogenic regulators elevated in patients with acute myocardial infarction, such as VEGF, angiopoietins and erythropoietin [11], or G-CSF [12]. Given such accumulated findings, TPO is intriguingly hypothesized to possess a preferential effect on EPC bioactivity, leading to a booster effect when co-administered in EPC transplantation.

Numerous studies in diverse cell types have demonstrated that TPO gives rise to biological functions of motility, survival, proliferation etc., through the orchestration of intracellular signaling pathways p44MAPK, JAK-STAT, or PI3K/Akt [13]. The Akt pathway has been revealed to be a key mediator for vasculogenic functions, such as migration, survival, differentiation, and proliferation in EPCs as well as ECs, concomitantly with phosphorylation of eNOS [14]. The mTOR (mammalian target of rapamycin)/p70S6kinase pathway, as the downstream effector of Akt activation, has also been indicated to regulate various biological functions of ECs for angiogenesis [15]. TPO is speculated to augment vasculogenic functions of EPC through the activation of the Akt/mTOR/p70S6kinase pathway. TPO has also been indicated to have angiogenic potential, not only by activating migration and tubulogenesis of human umbilical vein ECs (HUVECs) expressing c-Mpl *in vitro*, via the JAK-STAT pathway, but also by enhancing neovessel formation *in vivo* [16]. However, the association of PI3K/Akt and/or mTOR/p70S6kinase with TPO in an endothelial lineage for activating angio/vasculogenesis has not been evidenced as yet. Furthermore, the importance of sequential activation of the PI3K/Akt/mTOR/p70S6kinase pathway for EPCs remains to be elucidated, despite evidence that the mTOR/p70S6kinase pathway essential for producing EPCs from circulating ancestral CD133+ stem cells [17].

In the present study, we not only investigated the capability of TPO as a preferential mediator to ameliorate the efficacy of EPC transplantation for therapeutic vasculogenesis, but also assessed the intracellular mechanisms underlying the activities of EPCs.

2. Materials and methods

2.1. Materials

The following materials and antibodies were used: recombinant human TPO (KIRIN, JAPAN), recombinant human SDF-1α (PEPROTECH, France), LY294002 (SIGMA, USA), wortmannin (SIGMA, USA), rapamycin (Calbiochem, Germany), for FACS analysis; monoclonal anti-VEGF receptor-2 (KDR) (SIGMA, USA), purified mouse anti-human CD110 (c-Mpl), fluorescein isothiocyanate (FITC)-conjugated anti-human CD31, FITC-conjugate anti-human CD45, and phycoerythrin (PE)-conjugated CD31, CD133 (BD Pharmingen, USA), for immunohistochemistry; rabbit anti-human c-Mpl antibody (H-300) (SANTA CRUZE USA), Isolectin GSIB4 Alexa Fluor-488, Isolectin GSIB4 Alexa Fluor 594 (Molecular Probes, USA), for Western blotting analysis; anti-Actin (SIGMA, USA), phospho-mTOR (Ser2448), phospho-p70 S6 kinase (Thr421/Ser424), phospho-Akt (Ser473), Akt antibody (Cell Signaling, USA).

2.2. Ex vivo preparation of human cultured EPCs

Human cultured EPCs were cultured as described previously [1,2]. Briefly, total human peripheral blood mononuclear cells (PBMNC) isolated from healthy human volunteers by density-gradient centrifugation were cultured in human fibronectin coated Primaria tissue culture dishes (BD Falcon, USA), using 5% FBS-EBM-2 medium with EGMV-2 growth factor supplement (Clonetics, USA). Following the removal of suspended cells with PBS at day 4, adherent cells were cultured for 3 more days.

2.3. Cellular staining

After 7 days, cultured EPCs were detected by fluorescent staining of double positive cells with FITC-labeled *Ulex europaeus* agglutinin (UEA)-1 (Vector Lab, USA) and 1,1′-dioctadecyl-3,3,3,3′,3′-tetramethylindocarbocyyanine (DiI-I) labeled acetylated low density lipoprotein (DiI-acLDL Biomedical Technologies, USA). Cells were first incubated with DiI-acLDL at 37 °C for 4 h and later fixed with 1% paraformaldehyde for 10 min. After washing twice, the cells were reacted with FITC-conjugated UEA-1 (10 μg/ml) for 1 h. After staining, cells were observed with a fluorescence microscope (Olympus IX70, Japan). Cultured EPCs were also stained by mouse anti-human CD110 (c-Mpl) antibody with anti-mouse IgG1 Alex 594 as second antibody.

2.4. FACS analysis of cultured EPCs

Cultured EPCs from six healthy volunteer underwent FACS analysis not only to confirm their endothelial lineage but also assess c-Mpl expression, using PBS containing 2 mM EDTA, 1% BSA, and 0.1% $NaNO_3$. Cultured EPCs were stained with propidium iodide (PI) in order to exclude dead cells. The monoclonal antibodies for FACS staining were as follows: KDR (VEGFR-2), CD110 (c-Mpl), FITC-conjugated CD31, CD45 and PE-conjugated CD31, CD133. The cells were analyzed by FACS Calibur (BD Biosciences USA).

2.5. Migratory activity assay of cultured EPCs

To investigate EPC migratory activity, a modified Boyden chamber assay was performed using a 24-well microchemotaxis chamber with an 8 μm pore sized polycarbonate membrane (Corning Inc, USA), as described elsewhere [18]. The protocol was described in SM-I.

2.6. Proliferative activity assay of cultured EPCs

The assay for EPC proliferative activity effected by TPO was performed, according to the detail description in SM-II.

2.7. Survival assay of cultured EPCs

The assay for EPC survival effected by TPO was performed, according to detail description in SM-III.

2.8. RT-PCR for endothelial gene expression in cultured EPCs

The protocol for RT-PCR assay was described in SM-IV.

2.9. Lesion-targeted administration of TPO along with EPC transplantation in vivo

The impact of local administration of TPO after EPC transplantation on therapeutic neovasculogenesis was assessed in a hindlimb ischemic model, using athymic nude mice (Clea Japan Inc.) [2] for a TPO group or control (PBS). Soon after operative ligation of one femoral artery, 1 μg recombinant human TPO (rhTPO) in PBS (total 12 μl) per mouse was intramuscularly injected into ischemic thigh and lower muscles (total 3points), followed immediately by intravenous transplantation of (2.0×10^5 cells/mouse) cultured EPCs. To survey the transplanted EPCs incorporated into neovasculatures in ischemic muscles, four mice per group were injected with EPCs labeled with Cell Tracker CM-DiI (Molecular probes, USA), according to the manufacturers' protocol.