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Synergistic effect of sustained delivery of basic fibroblast growth factor and bone marrow mononuclear cell transplantation on angiogenesis in mouse ischemic limbs

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Abstract

Combined angiogenic therapies may be superior to single angiogenic therapy for treatment of limb ischemia. First, we investigated whether the angiogenic efficacy of basic fibroblast growth factor (bFGF) administration and bone marrow-derived mononuclear cell (BMMNC) transplantation can be enhanced by sustained delivery (SD) of bFGF and BMMNC transplantation using a matrix, respectively, in mouse ischemic limbs. Next, we investigated whether the angiogenic efficacy of combination of two angiogenic strategies is superior to either strategy alone. One day after surgical induction of mouse hindlimb ischemia, mice were randomized to receive either no treatment, daily injection (DI) of bFGF, SD of bFGF, BMMNC transplantation using culture medium, BMMNC transplantation using fibrin matrix (FM), or combination of SD of bFGF with BMMNC transplantation using FM. The SD of bFGF significantly increased the microvessel density, compared with DI of bFGF ($659 \pm 48/mm^2$ versus $522 \pm 39/mm^2$, $p < 0.05$). BMMNC transplantation using FM significantly increased the microvessel density, compared with BMMNC transplantation using culture medium ($523 \pm 103/mm^2$ versus $415 \pm 75/mm^2$, $p < 0.05$). Importantly, combination of bFGF sustained release with BMMNC transplantation using FM further increased the densities of microvessels and arterioles, compared to either strategy alone ($p < 0.05$). The SD method of angiogenic protein and cell transplantation using matrix potentiate the angiogenic efficacy of bFGF and BMMNC transplantation, respectively, for limb ischemia. In addition, the combined therapy of SD of bFGF and BMMNC transplantation synergistically enhances angiogenesis in mouse ischemic limb, compared to each separate therapy.

Keywords: Angiogenesis; Basic fibroblast growth factor; Cell transplantation; Fibrin; Limb ischemia

1. Introduction

Various methods of therapeutic angiogenesis have been used to induce collateral blood vessel development in clinical trials and animal models of limb and myocardial ischemia. The methods include angiogenic protein administration [1–8], endothelial progenitor cell (EPC) mobilization by cytokine [e.g., granulocyte colony-stimulating factor (G-CSF)] [9–11], and bone marrow-derived cell (BMC) transplantation [12]. The administration of recombinant angiogenic growth factors, such as basic fibroblast growth factor (bFGF) [1–3,5–8] and vascular endothelial growth factor (VEGF) [1,4], has been shown to enhance angiogenesis in ischemic diseases. BMC transplantation induced capillary regeneration and improved cardiac

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function [9]. Mobilization of EPC in bone marrow by cytokine induced the regeneration of vascular structure with a significant increase in capillary number and blood flow to ischemic site [10–12].

Recently, combination therapies have been shown to be synergistic in the induction of angiogenesis. Combined administration of VEGF and bFGF was reported to produce a greater and more rapid induction of angiogenesis than administration of either VEGF or bFGF alone [13]. The combination of platelet-derived growth factor-BB and bFGF was shown to synergistically induce angiogenesis and formation of long-lasting functionally stable vessels [14]. Furthermore, transplantation of VEGF-expressing myoblasts was found to enhance the angiogenesis with a larger number of mature capillaries than myoblast transplantation alone [15]. The combined transplantation of skeletal myoblasts and bone marrow stem cells was more effective in inducing angiogenesis than either type of cell transplantation alone [16].

In this study, we tested two hypotheses. The first hypothesis concerned whether the angiogenic efficacy of angiogenic protein and bone marrow-derived mononuclear cell (BMMNC) transplantation would be enhanced by sustained delivery (SD) of the protein and BMMNC transplantation using a matrix, respectively. The second hypothesis concerned whether combination of two strategies between angiogenic protein administration and BMMNC transplantation would be synergistic in angiogenesis induction. Thus, employing mouse hindlimb ischemia, angiogenic efficacy of the treatments was evaluated by immunohistological examinations and microvessel density determination in the ischemic areas.

2. Materials and methods

2.1. Mouse limb ischemia

Female C57BL/6J mice at 4 weeks of age (15–18 g body weight, Jungang Lab. Animal, Seoul, Republic of Korea) were anesthetized with xylazine (20 mg/kg) and ketamine (100 mg/kg). After the skin incision, the femoral artery and its branches were ligated with 5–0 silk (Ethicon, Sommerville, NJ, USA). Then, the external iliac artery and all of the above arteries were ligated. The femoral artery was excised from its proximal origin as a branch of the external iliac artery to the point distally where it bifurcates into the saphenous and popliteal arteries (Fig. 1) [17].

2.2. Isolation, characterization and labeling of BMMNC

Bone marrow cells were isolated from C57BL/6J mice as previous described [18]. For the flowcytometric analysis, BMMNCs were stained with FITC-conjugated anti-mouse CD34 monoclonal antibodies (BD Pharmingen, San Diego, CA, USA) and PE-conjugated anti-mouse AC133 monoclonal antibodies (eBioscience, San Diego, CA, USA) on ice for 20 min. The cells were washed twice with cold phosphate-buffered saline (PBS, pH 7.4, Sigma, St. Louis, MO, USA), fixed in PBS containing 1% (w/v) paraformaldehyde (Sigma), and analyzed with a fluorescence-activated cell sorter (FACS, BD Biosciences, San Jose, CA, USA). Prior to transplantation, BMMNCs were labeled with a fluorescent probe cell tracker (chloromethyl-1,1-dioactadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, CM-DiI, Molecular Probes, Eugene, OR, USA) that incorporates into the cell membranes [19]. Four weeks after cell transplantation, fluorescently labeled BMMNCs were detected using a confocal microscope (Eclipse E800, Nikon, Tokyo, Japan).

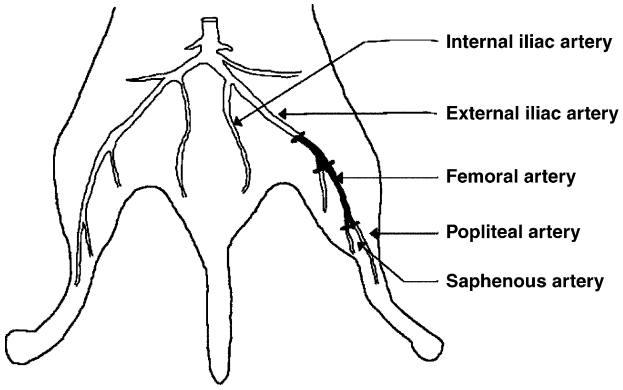

Fig. 1. Diagram of the mouse limb ischemic model development.

2.3. SD of bFGF using fibrin matrix (FM)

The profile of bFGF release from FM in vitro was determined as described previously [20]. The bioactivity of bFGF released from FM was assessed in vitro by determining its ability to stimulate proliferation of human umbilical vein endothelial cells (HUVECs) cultured in endothelial cell basal medium-2 (EBM-2, Cambrex Bio Science Walkersville, Inc., MD, USA) without serum. HUVECs (3×10^4 cells) were plated in each well of 6-well tissue culture plates, and cell number was determined with hemocytometer on day 0, 3, and 7. As a positive control, bFGF in free form was added daily to the HUVEC culture at the same concentration as that of bFGF released from the FM. HUVEC cultured in EBM-2 without bFGF served as a negative control. The medium was changed every 3 days, and the experiment was performed in triplicate.

2.4. Treatments of limb ischemia

One day after artery dissection surgery, mice were randomly assigned to one of six experimental groups ($n = 6$ for each group). No treatment was given in the control group. Five micrograms of bFGF was injected intramuscularly into gracilis muscle in medial thigh every day for 10 days (bFGF daily injection (DI) group). FM containing 25 μg of bFGF was injected intramuscularly into gracilis muscle in medial thigh on day 1 and 6 (bFGF SD group). BMMNCs (2×10^7 cells per mouse) were suspended in the culture medium (BMMNC/medium group) or FM (BMMNC/FM group) and injected intramuscularly into gracilis muscle in medial thigh. FM containing 25 μg of bFGF and BMMNCs (2×10^7 cells per mouse) and FM containing 25 μg of bFGF were intramuscularly injected into ischemic limbs on day 1 and on day 6, respectively (bFGF SD + BMMNC/FM group). In order to assess cell proliferation following the treatments, 5-bromo-2′-deoxyuridine (BrdU, 25 mg/kg body weight, Sigma) was subcutaneously administered postoperative every other day for 4 weeks.

2.5. Estimation of capillary density and arteriole density

Four weeks after the treatments, all mice were sacrificed. The ischemic limb specimens were retrieved from the injection site in medial thigh, fixed in formalin, embedded in paraffin, and sectioned at a thickness of 4 μm. For histological analysis, tissue sections were stained with hematoxylin and eosin (H&E). For capillary density assessment, the tissue sections were double stained with cyanine-conjugated anti-von Willebrand factor antibodies (vWF, Jackson Immuno Research, PA, USA) and