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Low-molecular weight heparin protamine complex augmented the potential of adipose-derived stromal cells to ameliorate limb ischemia

Satoko Kishimoto [a, b, *], Ken-ichi Inoue [a, b], Shingo Nakamura [c], Hidemi Hattori [c], Masayuki Ishihara [c], Masashi Sakuma [b, d], Shigeru Toyoda [b, d], Hideki Iwaguro [b, d], Isao Taguchi [b, e], Teruo Inoue [a, b, d], Ken-ichiro Yoshida [b]

[a] Research Support Center, Dokkyo Medical University, Mibu, Tochigi, Japan
[b] Center for Regenerative Medicine, Dokkyo Medical University, Mibu, Tochigi, Japan
[c] Division of Biomedical Engineering, Research Institute, National Defense Medical College, Saitama, Japan
[d] Department of Cardiovascular Medicine, Dokkyo Medical University, Mibu, Tochigi, Japan
[e] Department of Cardiology, Koshigaya Hospital, Dokkyo Medical University, Koshigaya, Saitama, Japan

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ABSTRACT

Background and aims: Heparin/protamine micro/nanoparticles (LH/P-MPs) were recently developed as low-molecular weight, biodegradable carriers for adipose-derived stromal cells (ADSCs). These particles can be used for a locally delivered stem cell therapy that promotes angiogenesis. LH/P-MPs bind to the cell surface of ADSCs and promote cell-to-cell interaction and aggregation of ADSCs. Cultured ADSC/LH/P-MP aggregates remain viable. Here, we examined the ability of these aggregates to rescue limb loss in a mouse model of hindlimb ischemia.

Methods: Unilateral hindlimb ischemia was induced in adult male BALB/c mice by ligation of the iliac artery and hindlimb vein. For allotransplantation of ADSCs from the same inbred strain, we injected ADSC alone or ADSC/LH/P-MP aggregates or control medium (sham-treated) directly into the ischemic muscles. Ischemic limb blood perfusion, vessel density, and vessel area were recorded. The extent of ischemic limb necrosis or limb loss was assessed on postoperative days 2, 7, and 14.

Results: Compared with the sham-treatment control, treatment with ADSCs alone showed modest effects on blood perfusion recovery and increased the number of α-SMA-positive vessels. Response to ADSC/LH/P-MP aggregates was significantly greater than ADSCs alone for every endpoint. ADSC/LH/P-MP aggregates more effectively prevented the loss of ischemic hindlimbs than ADSCs alone or the sham-treatment.

Conclusion: The LH/P-MPs augmented the effects of ADSCs on angiogenesis and reversal of limb ischemia. Use of ADSC/LH/P-MP aggregates offers a novel and convenient treatment method and potentially represents a promising new therapeutic approach to inducing angiogenesis in ischemic diseases.

1. Introduction

Adipose-derived stromal cells (ADSCs) are a type of mesenchymal stem cell (MSC) with numerous advantages in regenerative medicine, including multidirectional differentiation potential, strong proliferative capacity, low immunogenicity, and no legal or ethical issues associated with their use [1–4]. ADSCs can be induced *in vitro* to differentiate into various cell lineages, including adipocytes, osteocytes, chondrocytes, and endothelial cells [5]. Additionally, cultured ADSCs secrete significant amounts of angiogenic heparin-binding growth factors, such as basic fibroblast growth factor (b-FGF), hepatocyte growth factor (HGF), platelet-derived growth factors (PDGFs), and vascular endothelial growth factor (VEGF) as well as cytokines [6–8]. These stimulatory factors may contribute to the reported ability of ADSCs to regenerate damaged tissues, as demonstrated by transplanted human ADSC culture significantly stimulating angiogenesis [9]. Transplanted,

* Corresponding author. Research Support Center, Dokkyo Medical University, 880 Kitakobayashi, Mibu, Tochigi 321-0293, Japan.
E-mail address: skishi@dokkyomed.ac.jp (S. Kishimoto).

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fluorescent DiI-labeled ADSCs are partially incorporated into the regenerating granulation and epithelial tissues of *db*/*db* diabetic mice with some seeded ADSCs incorporated into blood vessels as endothelial progenitor cells as indicated by double labeling of microvessels with fluorescence (DiI) and CD31 [7] Thus, ADSC administration for tissue engineering represents a promising angiogenesis therapy. In fact, ADSC transplantation induces neovascularization in a mouse hindlimb ischemia model [10].

The clinical safety and efficacy of ADSC implantation have been reported in critical limb ischemia patients who were refractory to other treatment modalities [11]. However, the therapeutic efficacy of cell-based therapy is often limited by poor performance during engraftment, probably because of the rapid disappearance of grafted cells from the injection site [12,13]. Grafted cells are "alien" in terms of extracellular matrix constitution and hardly survive ectopic micro-environments. Thus, ADSC transplant together with an alternative cell-carrier may enhance ADSC survival.

We recently reported the development of low-molecular-weight heparin/protamine micro/nanoparticles (LH/P-MPs) as cell carriers for ADSC delivery into nude mice [14]. The LH/P-MPs bind to the ADSC through cell surface heparin-binding proteins such as integrins. Interactions between ADSCs and LH/P-MPs from ADSC/LH/P-MP aggregates (approximately several hundred μm). These aggregates strongly promote cellular viability *in vitro* and their injection into nude mice accelerates subcutaneous neovascularization [14].

In this study, using inbred mouse hindlimb ischemia model, we investigated the feasibility of the ADSC/LH/P-MP aggregates to ameliorate the impaired blood flow and the limb loss. One day after transplantation, ADSC increased systemic growth factors and cytokines but the levels dropped down 7 days later. Although LH/P-MP did not affect the initial surge of systemic growth factors, it augmented the angiogenic actions of ADSC *per se*. Thus, the combination of ADSC and LH/P-MP are a feasible strategy to ameliorate hindlimb ischemia.

2. Materials and methods

2.1. Preparation of LH/P-MPs

LH/P-MPs were synthesized as described previously [15,16]. Briefly, 0.3 mL of protamine sulfate solution (10 mg/mL; Mochida Pharmaceutical Co., Tokyo, Japan) was added dropwise to 0.7 mL of a Dalteparin sodium LH solution (6.4 mg/mL; Kissei Pharmaceutical Co., Tokyo, Japan), vortexed for 2 min, and washed twice with phosphate-buffered saline (PBS) and centrifuged at 4900 *g* for 5 min (MX-50; Tomy Seiko Co., Ltd, Tokyo, Japan) to remove non-reactants. The precipitates were resuspended in 1 mL of Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies Oriental, Tokyo, Japan). Upon drying, >6 mg of dry LH/P-MPs were obtained from each mL of resuspended LH/P-MPs solution. The final solution contained 60 mg of LH/P-MPs resuspended in 1 mL of DMEM.

2.2. Preparation of ADSCs and ADSC/LH/P-MP aggregates

All animal experiments adhered to the *Guidelines for Animal Experimentation of Dokkyo Medical University*, with all efforts made to minimize the animal numbers and suffering. ADSCs from inbred mice were prepared as previously described [7,14] with several modifications. Briefly, BALB/c mice (Japan SLC Co., Ltd, Shizuoka, Japan) adipose tissue from the inguinal region was removed, minced, transferred to C tubes (Miltenyi Biotec Corp., Tokyo, Japan), and digested with 0.1% collagenase type I (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.2% Dispase type II (Life Technologies) for 1 h at 37 °C. Digested tissue was mechanically and gently dispersed with a MACS Dissociator (Miltenyi Biotec Corp.) using the installed software program "m_brain01-02" every 10 min. The suspension was passes through a 100-μm filter (BD Falcon, NJ, USA), centrifuged at 420 *g* for 5 min (LC-200; Tomy Seiko Co., Ltd, Tokyo, Japan) and resuspended in DMEM. The cell concentration was determined using a hemocytometer. ADSCs (4×10^6 cells per mouse) were mixed with 160 μL of LH/P-MPs (60 mg/mL) and incubated for 1 h at 37 °C to prepare ADSC/LH/P-MP aggregates.

2.3. Evaluation of cell viability in culture

ADSCs were maintained in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin. To prepare ADSC/LH/P-MP aggregates, LH/P-MPs (1.4 mg of dried particles/mL) were added to a 15-mL polypropylene conical tube (BD Falcon, NJ, USA) with occasional shaking at 37 °C for the indicated time periods. After incubation, ADSCs alone or ADSC/LH/P-MP aggregates were removed from the medium, resuspended in 100 μL fresh medium with or without FBS, and transferred to a new 96-well tissue culture plate. To count living ADSCs, 10 μL WST-1 reagent (Cell Counting Kit, Dojindo, Kumamoto, Japan) was added, incubated for 1 h at 37 °C, and the optical density (OD) was read at 450 nm on an Immuno Mini plate reader (Nunc InterMed Japan, Tokyo, Japan) after an additional at 0.5, 10, 24, 48, and 96 h.

2.4. Analysis of ADSC-secreted growth factors and cytokines

ADSCs from inbred male BALB/c mice were plated on 10-cm plastic (control) or LH/P-MP-coated tissue culture plates, and ADSC/LH/P-MP aggregates were plated on 10-cm plastic suspension culture plates (each 1×10^6 cells per dish). LH/P-MP-coated tissue culture plates were coated for 3 h at 4 °C with 3 mL of LH/P-MP solution. The coating solution was then removed, and the plates were gently washed with PBS. ADSC-conditioned medium was collected 3 days after plating. At the end of culturing, supernatants were collected and stored at −80 °C until analysis. The presence of interleukin 6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), b-FGF, PDGF-bb, and VEGF was analyzed using the BioPlex system (Bio-Rad Laboratories Co. Ltd., Tokyo, Japan). HGF levels were measured in cell culture supernatants using an ELISA kit (Institute of Immunology Co. Ltd., Tokyo, Japan) [7,14].

2.5. Mouse hindlimb ischemia model and experimental protocol

Inbred male BALB/c mice aged 9–11 weeks (n = 18) were anesthetized with an intraperitoneal injection of 90 mg/kg ketamine hydrochloride (Ketalar; Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan) and 10 mg/kg xylazine hydrochloride (Celactal; Bayer Yakuhin, Ltd., Osaka, Japan). All mice underwent ligation of the right external iliac artery and hindlimb vein to produce right hindlimb ischemia [17]. Mice were randomly allocated into three groups (n = 6 each): sham-treated (DMEM) group, ADSC group (4×10^6 cells per mouse), and ADSC/LH/P-MP (ADSC/LH/P-MP aggregates) group. Treatments were injected at eight different sites (5×10^5 cells; 20 μL per site) on the adductor muscles of the ischemic limb immediately after surgery.

2.6. Hindlimb blood flow assessment

Hindlimb blood flow was analyzed using a laser Doppler blood perfusion imager (PeriScan PIM III; Perimed AB, Stockholm, Sweden) on postoperative day 0 (within 24 h of the operation) and days 2, 7, and 14. Depilatory cream was used to remove excess limb hair before imaging. Mice were placed on a heating plate at 38 °C to