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

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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,
fluorescent Dil-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 (Di) 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). LH/P-MPs (1.4 mg of dried particles/mL) were added to a 15-mL polypropylene conical tube (BD Falcon, NJ, USA) and centrifuged at 420 g for 5 min (LC-200; Tomy Seiko Co., Ltd, Tokyo, Japan). 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 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 passed 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 × 10⁶ 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, 1, 2, 4, 8, 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 × 10⁶ 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; Daichi 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 × 10⁶ cells per mouse), and ADSC/LH/P-MP (ADSC/LH/P-MP aggregates) group. Treatments were injected at eight different sites (5 × 10⁵ 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
minimize temperature variation during imaging. Blood perfusion was calculated from scanned images as perfusion units (PU) and serial changes in the ischemic (right) hindlimb were compared between groups. Changes in blood perfusion for the contralateral non-ischemic (left) hindlimb blood perfusion were also assessed to control for variation resulting from interference by ambient light and temperature.

2.7. Evaluation of hindlimb survival

Hindlimb survival was evaluated by morphologic observation on postoperative days 2, 7, and 14 and compared between groups. Limb ischemia was classified normal (limbs with a normal appearance), slight necrosis (toes or digits exhibited darkening), severe necrosis (entire foot exhibited darkening), and limb loss (auto-amputation) [18]. Hindlimb survival time was defined as the time post-surgery until the appearance of severe necrosis.

2.8. Analysis of growth factors and cytokines in the peripheral blood

Mouse peripheral blood was collected on postoperative days 1 and 7 and analyzed for IL-6, GM-CSF, b-FGF, PDGF-bb, VEGF and HGF concentrations using a multiplex Luminex kit (R&D Systems, Inc., Minneapolis, MN, USA). Magnetic beads were quantified using Luminex-200 (Luminex, Austin, TX, USA), and data were analyzed using xPONENT software version 3.1. Reference standards and patient samples were run in duplicate accordance with the manufacturer’s instructions. Growth factor and cytokine concentrations were determined from standard curves prepared on each plate and were expressed as pg/mL or ng/mL.

2.9. Immunohistochemistry

Ischemic thigh adductor skeletal muscle tissue samples were obtained on postoperative days 7 and 14 and processed for immunohistochemistry and blinded assessment of blood vessel density and area. The presence of collateral vessels that bypassed the occluded segment of the femoral artery was assessed by immunohistochemical staining with rabbit anti-α-smooth muscle actin (α-SMA) (Abcam Cambridge, MA) and evaluation of five random microscopic fields per slide by light microscopy (BX-53; Olympus Corp., Tokyo, Japan). α-SMA–positive vessel luminal area was quantified as the percentage of the total tissue area in a high power field (400×) using Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA). A threshold was selected and used to identify positive pixels on each slide. The percent area was calculated as the percentage of positive pixels relative to the total number of pixels in each view. Adjacent tissue sections were stained with hematoxylin and eosin [19].

2.10. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical significance was evaluated using unpaired Student’s t test for two-group comparisons. One-way analysis of variance (ANOVA) followed by Scheffe’s post-hoc test was used to compare ≥3 groups. Serial changes were evaluated using repeated-measures ANOVA. Probability values <0.05 were considered significant.

3. Results

3.1. Effect of LH/P-MPs on ADSCs

When ADSCs were suspended in DMEM in the presence of LH/P-MPs, LH/P-MPs induced ADSC/LH/P-MP aggregate formation within 3 h, with and without FBS (Fig. 1A). ADSC viability, as indicated by the OD value gradually decreased both with (1.64 ± 0.08 at 0.5 h to 0.37 ± 0.05 at 96 h, p < 0.01) and without (1.63 ± 0.07 at 0.5 h to 0.17 ± 0.03 at 96 h, p < 0.01) FBS. When ADSC/LH/P-MP aggregates were subsequently cultured, ADSCs maintained their viability for at least 96 h with FBS (1.56 ± 0.05 at 0.5 h to 1.66 ± 0.10 at 96 h), although the viability did decrease without FBS (1.65 ± 0.06 at 0.5 h to 1.22 ± 0.08 at 96 h, p < 0.01) (Fig. 1B).

3.2. ADSC production of growth factors and cytokines

Cultured ADSCs secreted substantial amounts of growth factors and cytokines over a 3-day period. Coating of the plastic tissue culture plate with LH/P-MPs significantly lowered the secreted growth factors and cytokines (Table 1), indicating that the material absorbs secreted proteins onto the plastic plate. On the other hand, the spheroid culture of ADSC/LH/P-MP further lowered the secreted growth factors and cytokines (Table 1). Thus, the formation of ADSC/LH/P-MP aggregates, rather than sheet-like structure, is and efficient way to absorb secreted proteins.

3.3. Blood flow recovery following injection of ADSC/LH/P-MP aggregates

Representative laser Doppler blood perfusion imaging (Fig. 2A) of hindlimb perfusion on postoperative days 0, 2, 7, and 14 was compared between treatment groups. On postoperative day 2, ischemic hindlimb blood perfusion was significantly lower than the contralateral control hindlimb in all groups. Although the sham-treated and ADSC groups showed lower blood perfusion until postoperative day 14, perfusion was remarkably restored in the ADSC/LH/P-MP group. Although ischemic limb blood perfusion was higher with ADSC than sham treatment on postoperative day 14 (84.95 ± 15.32 PU vs. 23.9 ± 16.51 PU, p < 0.05), it was far higher in the ADSC/LH/P-MP group (135.75 ± 25.66 PU, p < 0.05 for both comparisons). ADSC/LH/P-MP group ischemic limb blood perfusion was one-half that of the contralateral control hindlimb on postoperative day 7 (89.12 ± 26.37 vs. 173.61 ± 25.80, p < 0.05), but was nearly even with the control level (171.88 ± 20.86) on day 14 (Fig. 2B).

3.4. Prevention of limb loss by ADSC/LH/P-MP aggregate injection

Representative images of an ischemic limb in each group on postoperative days 0, 2, 7, and 14 are shown in Fig. 2C. In the sham-treated group, 4/6 mice (67%) developed limb loss, whereas the remaining 2 (33%) showed severe ischemia on postoperative day 14. In the ADSC group, 1/6 mice (17%) had limb loss and 3/6 mice (50%) had severe ischemia on postoperative day 14. Conversely, the ADSC/LH/P-MP group did not experience limb loss or severe ischemia. Moreover, the limbs of 4/6 mice (67%) appeared normal on postoperative day 14 (Fig. 2D). The median hindlimb survival time in the sham, ADSC, and ADSC/LH/P-MP groups was approximately 9, 15, and 28 days, respectively.

3.5. Peripheral blood growth factors and cytokines

Concentrations of IL-6, GM-CSF, b-FGF, PDGF-bb, VEGF, and HGF on postoperative days 1 and 7 are presented in Fig. 3. The factors GM-CSF ([43.88 ± 16.60 pg/mL [ADSC], 36.49 ± 5.74 pg/mL [ADSC/LH/P-MP], vs. 0.27 ± 0.03 pg/mL [sham-treated]; p < 0.01), b-FGF ([231.74 ± 71.18 pg/mL [ADSC], 340.75 ± 80.94 pg/mL [ADSC/LH/P-MP], vs. 95.03 ± 40.66 pg/mL [sham-treated]; p < 0.01), PDGF-bb ([486.06 ± 189.68 pg/mL [ADSC], 492.84 ± 115.38 pg/mL [ADSC/LH/P-MP], vs. 160.21 ± 113.2 pg/mL [sham-treated]; p < 0.01),
on day 14, p < 0.01. Staining was higher in the ADSC/LH/P-MP group (p < 0.01 on days 7 and 14) (Fig. 4C). The ADSC and ADSC/LH/P-MP groups showed an increase in the number of a-SMA-positive vessels and greater a-SMA-positive vessel lumen area compared with the sham-treated group on days 7 and 14 (5.2% ± 1.34% [ADSC], 7.86% ± 2.03% [ADSC/LH/P-MP], vs. 0.47% ± 0.16% [sham-treated] on day 7; 6.55% ± 1.26% [ADSC], 9.61% ± 2.90% [ADSC/LH/P-MP], vs. 0.64% ± 0.24% [sham-treated] on day 14; p < 0.01). Staining was higher in the ADSC/LH/P-MP than in the ADSC group (p < 0.01 on days 7 and 14) (Fig. 4C). These increases in a-SMA-positive vessel number and lumen area provide a histological basis for the increased blood flow and tissue viability in the ischemic limbs of ADSC and ADSC/LH/P-MP group mice.

4. Discussion

This study, demonstrated that LH/P-MPs promote cell-to-cell interaction and mouse ADSC aggregation. When cultured in suspension, ADSCs in aggregates but not ADSCs alone maintained their viability for at least 96 h in the presence of FBS. These results suggested that the ADSC/LH/P-MP aggregates enhance transplanted ADSC survival in vivo. In a mouse hindlimb ischemia model, ADSC/LH/P-MP aggregates were more effective than ADSCs alone at preventing hind limb loss, although ADSCs alone were more effective than the sham treatment. Compared to ADSCs alone, the ADSC/LH/P-MP aggregates stimulated greater angiogenesis as demonstrated by an increase in a-SMA-positive vessels and greater improvement in blood perfusion as demonstrated by laser Doppler blood perfusion imaging. The enhanced angiogenesis and blood perfusion achieved with ADSC/LH/P-MP aggregates likely resulted in the decreased ischemic limb loss. These results suggest that LH/P-MPs can augment the ability of ADSCs to ameliorate hindlimb ischemia.

Heparinoids, such as LH or heparan sulfate, are well-known co-factors that enhance the activities of heparin-binding growth factors [20]. LH/P-MPs are able to adsorb and enhance the activities of various heparin-binding growth factors and cytokines that are released from ADSCs, including IL-6, GM-CSF, b-FGF, PDGF-bb, VEGF, and HGF (Table 1). Similarly, LH/P-MPs show high affinities for interactions with FGF-2, HGF, and various heparin-binding growth factors derived from platelet-rich plasma (PRP) [8]. Also they were efficiently adsorbed by LH/P-MPs, enhancing their activities and protecting those growth factors from inactivation by acidic or high heat environments and from protease degradation in vitro. Furthermore, LH/P-MPs bind to the ADSC cell surface through heparin-binding cell surface proteins such as Integrins. The interaction of ADSCs with LH/P-MPs results in ADSC/LH/P-MP aggregate formation. Aggregate formation appear to promote cellular viability in vitro [14]; thus, subcutaneous injection of mouse
ADSC/LH/P-MP aggregates could stimulate cell proliferation and migration, resulting in neovascularization in vivo [21]. ADSCs can potentially release multiple angiogenic growth factors that can be efficiently immobilized onto LH/P-MPs [8] or LH/P-MP-coated culture plates [22]. PRP/LH/P- MPs prevented limb loss in an adult BALB/c- nu/nu male mouse-induced ischemic hindlimb model [23]. Thus, LH/P-MPs can enhance the angiogenic effects of ADSCs through their ability to immobilize and to activate various angiogenic factors.

The suggested angiogenic mechanism involves not only ADSCs per se, but also endogenous inflammatory cells to induce the release of multiple angiogenic factors [24,25]. Next, these angiogenic factors are adsorbed onto LH/P-MPs, permitting their stabilization and bioavailability. The stability and availability of angiogenic factors are therefore enhanced by LH/P-MPs. Finally, the activated angiogenic factors markedly induce angiogenesis.

Surgical injury induced a systemic response on postoperative day 1 in all groups other than in the control group. However, the peripheral blood levels of five factors (GM-CSF, b-FGF, PDGF-bb, VEGF, and HGF) were significantly higher in the ADSC and ADSC/LH/P-MP groups than in the sham-treated group and all had decreased on day 7. Thus, we can explain the underlying mechanisms in two steps. First, ADSC transplantation evokes a potent local and systemic inflammatory response. Immune cells are recruited from the bone marrow to locations throughout the body. The burst of growth factor and cytokine production may exceed the capacity of LH/P-MPs to adsorb them. At this stage, the effects of the LH/P-MPs were not significant (Fig. 3). Next, after the inflammation subsides, the LH/P-MPs sustain the stability of local growth factors and cytokines, enabling prolonged angiogenesis. ADSC is not the sole player to produce the growth factors and the ADSC augments immune cell mobilization. ADSC/LH/P-MPs, on the other hand, is critical for the second step (coordinated repair) because of two beneficial effects (sustained survival of ADSC and stabilization of released growth factors). Therefore, the combination of multiple growth factors may be theoretically sufficient to mimic the second step. These mechanisms might explain how LH/P-MPs augmented the ADSC potential to ameliorate hindlimb ischemia.

Heparin is used clinically as an antithrombotic agent, but at doses that are limited by strong intrinsic anticoagulation to avoid the potential for severe bleeding complications. However, LH shows much lower anticoagulation activity than native heparin. Protamine is also in clinical use to reverse heparin anticoagulant activity following cardiopulmonary bypass or with heparin-induced bleeding [15]. No bleeding complications were observed among mice injected with LH/P-MPs in this study.

Peripheral arterial disease (PAD) is a major healthcare problem in an aging society [26]. PAD commonly affects the arteries...
supplying the lower limbs. Because PAD is the most common cause of atherosclerotic disease, it often complicates other atherosclerotic diseases such as coronary artery disease and stroke. It is well recognized that patients with PAD will probably die from a
cardiovascular disease [27]. Advanced PAD has been termed critical limb ischemia. In critical limb ischemia patients, limb loss can occur when the ischemia is not resolved by either percutaneous or surgical revascularization procedures [28,29]. New approaches involving stem cell therapy are being investigated. Bone marrow-derived stromal cells (BMSCs) can improve limb ischemia after injection and have prevented limb loss in experimental limb ischemia models [30,31]. Studies have demonstrated the effectiveness of autologous BMSCs in patients with ischemic limbs [32–34]. MSCs are known to induce neovascularization in a hindlimb ischemia model [35].

Adipose tissue is abundant in the human body and is constantly reorganized through angiogenesis. Therefore, this tissue is an ideal source of angiogenic MSCs. It has been shown that ADSCs have characteristics similar to those of BMSCs [36,37]. The implantation of cultured ADSCs in the mouse hindlimb ischemia model showed proangiogenic activity [10]. Additionally, the safety and effectiveness of autologous ADSCs implantation in critical limb ischemia patients was reported [11]. Recently, an automated cell-processing system for ADSC isolation has been developed, and its safety and reproducibility have been assessed [38]. Intramuscular injection of ADSCs/LH/P-MPs may provide a new therapeutic opportunity whereby ADSCs enhance angiogenesis and vasculogenesis in ischemic tissues with impaired blood flow. ADSCs/LH/P-MPs preparation is simple, and ADSCs/LH/P-MPs can be readily and directly administered into the ischemic region. Because all components are in a clinical use and have already passed a significant number of toxicity and other tests, their safety is well known. Drug repositioning (also referred to as drug repurposing), the process of finding new uses of existing drugs, has been gaining popularity in recent years. Repurposed drugs such as these can bypass much of the early cost and time needed to bring a drug to the market [39].

5. Conclusion

Intramuscular injection of inbred mouse-derived ADSCs into mouse ischemic hindlimbs promoted neovascularization and prevented ischemic limb loss. LH/P-MPs present a potentially an excellent biomaterial for immobilizing, retaining, and gradually releasing various heparin-binding growth factors from ADSCs to optimally sustain vascularization. Additionally, LH/P-MPs can act as an efficient cell carrier in vivo. In the future, ADSC pluripotency and their secreted growth factors may be utilized in regenerative medicine. The approach presented here using ADSCs/LH/P-MPs may represent a valuable treatment option to enhance therapeutic angiogenesis for tissue regeneration and to treat ischemic disease.

Conflict of interest

The authors declare that they have no conflict of interest.

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