Human Adipose Tissue-Derived Mesenchymal Stem Cells Improve Postnatal Neovascularization in a Mouse Model of Hindlimb Ischemia

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Key Words

Vasculogenesis • Endothelium • Human adipose stromal cells • Hindlimb ischemia

Abstract

Background/Aim: It has been reported that adipose tissue contain progenitor cells with angiogenic potential and that therapy based on adipose tissue-derived progenitor cells administration may constitute a promising cell therapy in patients with ischemic disease. In this study we evaluated the effect of culture-expanded mesenchymal stem cells (MSC) derived from adipose tissue on neovascularization and blood flow in an animal model of limb ischemia in immunodeficient mice. **Methods:** MSC were cultured from human adipose tissue by collagenase digestion. Hindlimb ischemia was created by ligating the proximal femoral artery of male nude mice. Human adipose tissue stromal cells (hADSC) were transplanted one day or 7 days after ligation. **Results:** During culture expansion of hADSC CD34 expression was downregulated. The laser Doppler perfusion index was significantly higher in the CD34(-), Flk-1(-), CD31(-) ADSC-transplanted group than in the control group, even when cells were transplanted 7 days after

hindlimb ischemia. Histological examination showed that hADSC transplantation recovered muscle injury and increased vascular density, compared with the control group. The effect of hADSC was correlated with the number of transplanted cells, but not with the ratio of CD34 expression. In vitro, hADSC can form vessel-like structure and express von Willibrand Factor. Conditioned media from hADSC increased proliferation and inhibited apoptotic cell death in of human aortic endothelial cells. **Conclusion:** This study showed that hADSC can be an ideal source for therapeutic angiogenesis in ischemic disease.

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Introduction

Peripheral vascular disease is a major health care problem in an aging society [1]. An important compensatory response to atherosclerotic obstructive arterial disease is collateral development, a complex process requiring that multiple genes coordinately express their products in an appropriate time-dependent manner [2]. However, the natural capacity of collaterals to remodel and enlarge to compensate for the reduced flow

that occurs after occlusion of a major artery is rarely sufficient to restore maximal flow capacity to levels required under various stress-conditions. In the late stages of peripheral vascular disease, progression of tissue hypoperfusion results in ischemic ulceration and gangrene. Unfortunately, amputation is required in more than a third of these patients. Rapid revascularization of injured, ischemic, and regenerating organs is essential for the restoration of their physiological function.

Although several protein and gene-based strategies have succeeded in enhancing collateral development in animal models of ischemia, clinical studies thus far have been disappointing [3, 4]. Given that the natural response to tissue ischemia is such a complex process, the delivery of a single growth factor may be too simple an approach. Thus, a great deal of interest has arisen in the potential of cell-based strategies in augmenting collateral responses [5, 6]. Recent findings indicate that hemangioblasts or more mature endothelial progenitor cells (EPC) persist into adult life, at which time they may circulate, differentiate, and contribute to the formation of new blood vessels [7, 8]. The angiogenic switch initiates the neovascularization process by recruiting EPC that assemble into neovessels. Because tissue injury disrupts the permissive environment necessary for the recruitment of EPC, introduction of exogenous progenitors may facilitate revascularization. To date, umbilical cord blood and bone marrow have represented the main sources of EPC in postnatal life [9]. Transplantation of bone marrow-derived mononuclear cells (MNC) has been shown to induce therapeutic neovascularization in critical limb ischemia [9, 10]. However, EPC expansion on a large scale is still technically demanding.

Bone marrow stromal cells (BMSC), a representative mesenchymal stem cell (MSC) possess pluripotency and differentiate into osteoblasts, chondrocytes, neurons, skeletal muscle cells, endothelial cells and vascular smooth muscle cells [11-13]. BMSC have an adherent nature and are expandable in culture. Thus, it would be easy to obtain a sufficient number of BMSC for cell therapy. BMSC have been shown to form capillary-like structures in an in vitro Matrigel assay [14] and BMSC transplantation has been shown to induce neovascularization in a rat model of hindlimb ischemia [15]. However, BMSC transplantation requires harvesting a large amount of bone marrow under general anesthesia, which may impose a load on some patients with severe complications such as myocardial ischemia, heart failure, cerebral disease, or renal failure.

Development of the capillary network is required to ensure adipose tissue remodeling [16, 17] and antiangiogenic agents promote adipose tissue loss [18], underlining a crucial link between adipose cells and the capillary network. Adipose lineage cells have been shown to release potent angiogenic factors such as monobutyril, vascular endothelial growth factor (VEGF), and leptin [17, 19, 20], and to differentiate into endothelial cells in vitro and augment recovery of perfusion in hindlimb ischemia mouse model [21]. However, the identity of the cells is still controversial. Miranville et al. [22] and Rehman et al. [23] showed CD34(+) cells in human adipose tissue have proangiogenic effects, and Cao et al. [21] reported that Flk1(+), CD34(-) cells also have similar effects on hindlimb ischemia model.

Adipose tissue have MSC, which can differentiated into bone, fat, muscle, and cartilage cells [14, 22, 24-26]. It has been reported that MSC derived from adipose tissues have similar characteristics of BMSC [27-29]. In this study, we determined whether adipose tissue-derived MSC can differentiate into endothelial cells in vitro and have proangiogenic action in hindlimb ischemia of nude mice.

Materials and Methods

Culture of mesenchymal stem cells

All protocols involving human subjects were approved by the Institutional Review Boards of Pusan National University. hADSC were isolated according to the methods described in the previous studies [27]. To isolate hADSC, adipose tissues were washed with phosphate-buffered saline (PBS), and tissues were digested at 37°C for 30 min with 0.075% type I collagenase. Enzyme activity was neutralized with α -modified Eagle's medium (α -MEM), containing 10% fetal bovine serum (FBS) and centrifuged at 1,200 x g for 10 min to obtain a pellet. The pellet was incubated overnight at 37°C/5% CO₂ in control medium (α -MEM, 10% FBS, 100 units/ml of penicillin, 100 μ g/ml of streptomycin). Following incubation, the tissue culture plates were washed to remove residual nonadherent cells and maintained at 37°C/5% CO₂ in control medium. When the monolayer of adherent cells has reached confluence (P0), cells were trypsinized (0.25% trypsin; Sigma), resuspended in α -MEM containing 10% FBS, and subcultured at a concentration of 2,000 cells/cm². For most experiment, we used 3rd-5th passages of hADSC.

P0 cells were resuspended in phosphate-buffered saline (PBS), 5 mM EDTA. Cells were incubated at 4°C with magnetic beads which were coated with a monoclonal antibody specific for human CD34, and processed through a MACS magnetic separation column (Miltenyl Biotech) to separate CD34+ cells.

Flow cytometry analysis

To characterize the phenotypes of adipose-derived cells, flow cytometry analysis was performed. At least 50,000 cells (in 100 μ l PBS/0.5% BSA/2 mmol/L EDTA) were incubated with fluorescence-labeled monoclonal antibodies against human Flk-1, CD90, CD31, CD34, CD44, CD45 (BD Biosciences) or the respective isotype control (1/20 diluted, 4°C, 30 minutes). After washing steps, the labeled cells were analyzed by flow cytometry by use of a FACS Calibur flow cytometer and the Cell Quest Pro software (BD Biosciences).

Hindlimb ischemia model and cell transplantation

The neovascularization capacity of hADSC was investigated in a murine model of hindlimb ischemia in 8 week-old Balb/C nude mice (Sam: TacN(SD)fbr). To produce hindlimb ischemia, animals were anesthetized by pentobarbital sodium (0.5mg/g). The proximal portion of the femoral artery including the superficial and the deep branches were ligated twice with 7-0 silk suture, and the overlying skin was closed. After 24 hours, injection of hADSC was performed intramuscularly by injecting cells resuspended in phosphate buffered saline (PBS) at three different sites of the ischemic leg. For the tracing of transplanted hADSC, cells were marked with the fluorescent CM-DiI dye (Molecular Probes) before cellular transplantation.

After 2 weeks, cutaneous blood flow was measured by a laser Doppler blood flow meter (Laser Doppler Perfusion Imager System, OMEGA FLOW FLO-CI, OMEGA WAVE, INC.). Before scanning was initiated, mice were placed on a heating plate at 37°C.

Histological evaluation

Mice were killed after blood flow was determined by a laser Doppler blood flow meter, and perfused with 4% (w/v) paraformaldehyde (Sigma). The hindlimb ischemic muscles were removed and fixed in neutral buffered 4% (w/v) paraformaldehyde at 4°C for 24 hours prior to embedding in paraffin and sectioning. Transverse muscular sections (5 μ m) were deparaffinized and then stained with hematoxylin and eosin (H-E). The slides were examined at 200 x magnification.

Immunohistochemistry

Paraffin sections (5 μ m) taken from the ischemic and nonischemic hindlimb were deparaffinized, and then subjected to antigen retrieval. Blocking was performed with 2% BSA for 1 hour at room temperature to block non-specific binding. Staining for endothelial cell of blood vessels was performed with rabbit anti-human von Willibrand Factor (vWF) polyclonal antibody (1:200, Chemicon international) and followed by incubation with FITC-conjugated anti-rabbit polyclonal antibody (1:100, Jackson ImmunoResearch laboratory). The specimens were observed under a Leica TCS SP2 confocal microscope.

Preparation of Conditioned Media From hADSC

hADSC were cultured and expanded on tissue culture plates in EGM-2-MV medium and used for the experiments at passages 0 through 3. At 90% confluence, culture medium was switched to EBM-2 and placed for 48 hours. At the end of the

incubation period, the conditioned media from hADSC were collected (hADSC-CM).

Cytokine array analysis

At 90% confluence, the culture media of hADSC were switched to serum free α -MEM and were placed for 24 hours. At the end of the incubation period, the conditioned media from hADSC were collected for analysis of cytokine expression. Assay of Human Cytokine Expression was performed using RayBio® Human Angiogenesis Antibody Array C Series 1000 (RayBiotech) according to the manufacture's instructions. The detected signals were quantified by gel documentation system (UVItec, Cambridge).

Endothelial Cell Proliferation Assay

Human aortic endothelial cells (Cambrex) at passages 7 or less were seeded at a density of 10,000 cells per well in a 12-well plate and then switched for 24 hours to EBM-2/5% FBS medium, which limits cell growth, because of the absence of supplemental growth factors. On the following day, the EBM-2/5% FBS medium was then replaced with 50% fresh EBM-2/5% FBS medium (control medium) and 50% hADSC-CM. Cell numbers were determined by a hemocytometer 3 days after the treatment of conditioned media.

To determine Brdu incorporation, human aortic endothelial cells were incubated with 2.5M bromodeoxyuridine (Brdu, sigma) for 24 hours and then Brdu-labeled cells were detected by immunocytochemistry with anti-rat Brdu antibody.

Endothelial Cell apoptosis assay

To assess the effects of hADSC-CM on endothelial cell apoptosis, human aortic endothelial cells at passages 7 or less (Cambrex) were grown in 12 well plate for 3 days and then incubated for 24 hours with 3g/mL cycloheximide (Sigma) and 10 ng/mL tumor necrosis factor- α (TNF- α) (Sigma) in the EBM-2 (control medium) or hADSC-CM. After incubation for 24 hours, endothelial cells were fixed in 4% paraformaldehyde, and terminal dUTP nick-end labeling (TUNEL) (In situ cell death detection kit, Roche) was performed for detection of apoptotic nuclei according to the manufacturer's instructions. Randomly selected microscopic fields (n=10) were evaluated to calculate the ratio of TUNEL positive cells to total cells.

In vitro tube formation assay

Thawed Matrigel (Chemicon) was added to 96-well plates and allowed to solidify at room temperature for 1 hour. hADSC were seeded 1x10⁴ cells per well and were induced to differentiate into endothelium by plating in Matrigel-coated 96 well plate with EGM-2-MV for 5 days. Matrigel with hADSC was dispersed with dispase II (Roche) and the retrieved cells were allowed to attach to the coverslip for 1 day. vWF expression was determined by immunocytochemistry.

Immunocytochemistry

The cells were fixed with 4% paraformaldehyde for 15 minutes at 4°C and permeabilized with 0.3 % triton X-100 for 10 minutes. Samples were blocked for 1 hour with 8% bovine

Fig. 1. Flow cytometry histogram of P0 cells isolated from human adipose tissue.

albumin serum (BSA) at RT. Primary antibodies were diluted in PBS with 2% BSA and were present at 4°C overnight. Secondary antibodies were diluted in PBS with 2% BSA and incubated for 1 hour at RT. Primary antibodies dilutions were as follows: 1:500 for vWF (Chemicon), 1:500 for CD34, and 1:40 for BrdU. Secondary reagents were FITC-conjugated anti-rabbit (Jackson ImmunoResearch laboratory), TRITC-conjugated anti-mouse (Jackson ImmunoResearch Laboratory) and FITC-conjugated anti-rat (Jackson ImmunoResearch Laboratory) antibodies.

To detect BrdU-positive cells, BrdU labeled hADSC were fixed with phosphate-buffered 4% (v/v) paraformaldehyde and treated in 50% formamide solution, 40% distilled water and 10% 20x SSC solution for 2 hours at 65°C followed by treatment in 2 N hydrochloric acid for 30 minutes at 37°C and then treated 100mM sodium borate solution for 10 minutes at room temperature. And then the following steps were same.

Statistical analysis

All results are presented as mean $\pm$ SEM. Comparisons between groups were analyzed by use of t test (2-sided) or ANOVA for experiments with more than 2 subgroups. Post hoc range tests and pairwise multiple comparisons were performed with the t test (2-sided) with Bonferroni adjustment. Probability values of $P < 0.05$ were considered statistically significant.

Results

Surface marker expression of hADSC

Adipose tissue-derived cells have been reported to express CD34 [22, 23]. However, when we cultured

Fig. 2. Measurement of expression of CD34 on hADSC cultured in α -MEM medium over time. After initial plating, cells were passaged three times by 1:3 dilution during the experimental period. Data represent mean ($n = 4$).

MSC from one gram of adipose tissue, it took 7-8 days to get a confluent growth in a 60 cm² dish, and the cultured cells did not express CD34. To get a confluent cell growth in a 60 cm² dish from small number of tissues, cells should divide several times. Therefore, we supposed that CD34 expression in hADSC is dependent on the number of population doubling. To test this possibility, we isolated

Fig. 3. Immunocytochemistry of CD34 on MACS-sorted cells (A) and colony derived from single CD34(+) cells (B). CD34(+) cells from P0 cells were sorted by MACS, and plated for CFU assay. MACS-sorted cells and the colonies derived from single CD34(+) cells were stained with CD34(+) antibody.

Fig. 4. Representative photograph of PBS-injected or hADSC-transplanted mice. Hindlimb ischemia was induced by femoral artery ligation in nude mice, and animals were treated with PBS (A) or 5×10^5 ADSC (passage 3-5). Photos of PBS (A) or hADSC (B) transplanted ischemic hindlimbs were taken on day 21 after transplantation.

cells from 100 ml of lipoaspirates by collagenase digestion, and the whole cells were plated on a 60 cm² culture dish. Cells reached at confluent state at the 2nd day after plating (P0). The adherent cells showed a fibroblast-like morphology when observed under a light microscope. The morphology was maintained through repeated subcultures under non-stimulating conditions. To characterize the phenotypes of adherent adipose-derived cells, flow cytometry was performed. P0 Cells showed 96.25% CD34 positive, 87.63% CD44, 98.27% CD90, but did not express other hematopoietic and endothelial markers (CD45, CD31 and Flk-1) (Fig. 1). Then, we determined the percentage of CD34(+) cells during the following passages. The percentage of CD34 positive cells was dramatically decreased during subsequent cultivations (Fig. 2). After 6-7 population doublings, CD34 expression in most cells was lost and cells were expanded logarithmically. To determine whether decrease of CD34 expression was resulted from downregulation of CD34 transcription or from the selection of CD34(-) subsets,

we sorted CD34(+) cells by MACS and performed colony forming unit assay. When we plated 60 hADSC on a 100 mm culture dish, 63% of cells formed colony. Immunocytochemistry of colonies showed that all colonies derived from CD34(+) cells were not stained with CD34 antibody (Fig. 3).

In vivo transplantation of hADSC in hindlimb ischemia model

The angiogenic potential of hADSC was first assessed in hindlimb ischemia model of nude mice. Mice receiving PBS experienced severe ischemic damage resulting in limb contracture and a 60% incidence of autoamputation by day 28. However, mice receiving hADSC displayed normal hindlimb appearance with no autoamputation (Fig. 4). The relative blood flow index obtained from normal mice was 47 ± 4.5 (n=10). Blood perfusion of the ischemic hindlimb was considerably impaired. The intramuscular injection of 1×10^6 cultured hADSC at the following day after proximal femoral artery

Fig. 5. Representative histological photographs (A,B,C) and vWF immunohistochemistry (D,E,F) of tissue sections taken from control hindlimb (A,D) and ischemic hindlimb of PBS-injected (B,E) or hADSC-transplanted group (C,F). Hindlimb ischemia was induced by femoral artery ligation in nude mice, and animals were treated with either PBS or different number of hADSC (p3-5) 1 day after ligation. The skeletal muscles of hindlimb were taken 14 days after ligation. Paraffin-embedded tissue sections were stained with H-E and vWF antibody. (B) Morphology of muscles in mice that showed signs of massive tissue degeneration after ischemia. Note swollen muscle fibers and the presence of numerous granulocytes and neutrophils indicative of tissue inflammation. (C) Regenerating muscle after ischemia in hADSC-injected mice. Note the smaller size of the fibers and the presence of centrally located nuclei (arrow). Arrow shows that injection of hADSC caused a significant increase of muscle regeneration compared with PBS injected group.

Fig. 6. Effect of cell numbers (A) and CD34 expression (B) on blood flow of ischemic hindlimb. Hindlimb ischemia was induced by femoral artery ligation in nude mice, and animals were treated with either PBS or different number of hADSC (p3-5) 1 day after ligation. Quantitative analysis with laser Doppler was performed over time on day 14. To determine the percentage of CD34(+) expression on blood flow, 10^6 CD34(+) (P0) or CD34(-) (p3-p5) hADSC were transplanted 1 day after ligation. Data represent mean $\pm$ SEM (n=6). *P<0.05, compared with PBS injected group.

Fig. 7. Effect of transplantation period of hADSC on blood flow of ischemic hindlimb. Hindlimb ischemia was induced by femoral artery ligation in nude mice, and animals were treated with either control medium, 5×10^5 hADSC 1 or 7 days after ligation. Quantitative analysis with laser Doppler was performed over time on days 0, 7, 14 and 21. Data represent mean $\pm$ SEM (n=6), *, P<0.05, compared with PBS-injected group, #, p<0.05, compared with hADSC transplantation 1 day after ligation.

Fig. 8. Representative photograph of a RayBiotech C1000 angiogenesis array hybridized with hADCS-CM. Black dot represent positive signals. P, Positive control; N, Negative control; B, Blank.

ligation significantly improved cutaneous blood flow (43 ± 3.2 in hADSC group vs 19 ± 2.5 in PBS group, $p < 0.05$, $n=10$). Histological examination of ischemic limb showed that hADSC transplantation protected atrophic and necrotic damage of muscle. Immunohistochemistry with vWF antibody showed that a large number of capillaries surrounding large vessels were detected in the ischemic muscle of the hADSC group, compared with PBS group (Fig. 5).

To examine the relationship between the number of transplanted cells and their angiogenic potential, different number of hADSC (10^5 cells to 10^6 cells per animal) was transplanted into the ischemic thigh muscle ($n=10$ each). LDPI index increased according to the number of transplanted cells (Fig. 6A). We next compared proangiogenic effect of CD34(+) or CD34(-) hADSC in hindlimb ischemia model of nude mice. There was no significant difference in the blood flow 2 weeks after transplantation between CD34(+) (P0) and CD34(-) hADSC (P5) (Fig. 6B).

In most clinical situation, autologous hADSC

transplantation at the following day after ischemia was not possible. Therefore, we determined whether hADSC transplantation at 7 days after ischemia was also effective. Without cell transplantation, contracture of both ischemic hindlimb was observed 7 days after ischemia. Two weeks after transplantation, hindlimb contracture was relieved at the transplanted side, whereas necrotic injury at the nontransplanted side was progressed. When ADSC were transplanted at 1 week after ligation, the hindlimb blood flow was recovered to the nonischemic level at one week after transplantation, compared with a partial recovery when hADSC were transplanted at 24 hours after ligation (Fig. 7).

Analysis of cytokine production and effect of hADSC conditioned media on proliferation and apoptosis of endothelial cells

Two potential mechanisms could explain this proangiogenic effect. One is the release by hADSC of angiogenic growth factors, and the other is a direct contribution of injected cells by their incorporation into

Fig. 9. Effect of conditioned medium from hADSC on proliferation (A, B) and apoptotic cell death (C) of human aortic endothelial cells. Cell proliferation was determined by direct counting of cell number 3 days after plating (A) and Brdu incorporation (B). Data represent mean $\pm$ SEM (n=4). *, p<0.05 compared with control. (C) Human aortic endothelial cells were incubated with cycloheximide and TNF- α for 24 hours. Apoptosis was analyzed by TUNEL assay. Data are shown as percentage of apoptotic endothelial cells (mean $\pm$ SEM, n=4). *, p<0.05 compared with control.

Fig. 10. Endothelial differentiation of hADSC in Matri-gel. (A) phase contrast micrographs of a vessel-like structure formed by hADSC. (B,C) Immunocytochemistry of differentiated hADSC. hADSC cultured in Matri-gel for 2 days were dissociated with the treatment of dispase. The dissociated hADSC were stained with vWF antibody and examined by fluorescent microscopy.

regenerative vessels.

To determine production of proangiogenic cytokines from hADSC, cytokine contents in the conditioned media of hADSC was determined by RayBiotech angiogenesis cytokine array. Strong signals were detected in GRO, placental growth factor, EBN-78, MCP-1, IL-6, IL-8, TIMP-1, TIMP-2, MMP-1, MMP-9 and uPAR (Fig. 8).

To examine whether hADSC-CM exhibited biological effects relevant to collateral remodeling, endothelial cell proliferation was determined by measurement of cell number and Brdu incorporation. hADSC-CM significantly enhanced proliferation of human aortic endothelial cells over control (Fig. 9A,B). TUNEL assay showed that combined treatment of cycloheximide

and TNF- α in human aortic endothelial cells induced apoptotic cell death. The addition of hADSC-CM protected apoptotic death of human aortic endothelial cells (Fig. 9C).

Transdifferentiation of hADSC to endothelial cells

To induce hADSC into functional endothelial cells in vitro, hADSC were cultured with 50 ng/ml VEGF and 10 ng/ml b-FGF on the Matrigel. Light microscopy observation over a period of 48 hours shows that vascular network was formed by hADSC after incubation with endothelial differentiation medium. Immunocytochemistry of hADSC that were recovered from matrigel by the

Fig. 11. Immunohistochemistry on paraffin sections of ischemic muscles injected with CM-DiI labeled hADSC. Ischemic hindlimb muscles were taken from hADSC-transplanted mice 4 weeks after ligation. Tissue sections were incubated with vWF antibody and FITC-labeled secondary antibody. Merger (yellow) of hADSC (red) and vWF (green) staining of endothelial cells in muscles of mice injected with CM-DiI labeled hADSC suggest hADSC differentiation into vascular endothelial cell (arrow).

treatment of dispare revealed that these cells were strongly stained by antibodies directed against vWF (Fig. 10).

Red fluorescence (CM-DiI)-labeled hADSC was detected in the interstitial tissues between muscle fibers 3 weeks after transplantation (Fig. 11). CM-DiI-positive cells expressed vWF, an endothelial marker, in ADSC groups. Quantitative analysis demonstrated that the number of CM-DiI/double positive cells was section, indicating that small proportion of transplanted cells can differentiate into endothelial cells and directly contribute to vascular regeneration. The ratio of vWF expressing cells among CM-DiI labeled cells in serial sections of tissues was less than 1%.

Discussion

Stem cell therapy is a promising approach in cardiovascular medicine. There are now reports [30] of clinical studies in which EPC have been used for neovascularization of ischemic organs. However, the paucity of EPC in the circulation, combined with their putative functional impairment in pathological conditions, limits their therapeutic application. Thus, the identification of suitable sources of angiogenic cells represents a

challenge for therapeutic angiogenesis of ischemic tissues. The use of umbilical cord blood, mobilization of EPC by growth factors, or the local infusion of suspensions of autologous bone marrow cells have been suggested as ways in which to circumvent this problem. However, traditional bone marrow procurement is painful and yields a low number of mesenchymal stem cells [31].

Human adipose tissue consists of adipocytes and cells composing the so-called stromal vascular fraction (SVF). On the basis of immunohistochemistry, the SVF from human mammary and visceral adipose tissue has been reported to contain EC as well as noncharacterized stromal cells, blood cells, and tissue macrophages [32]. hADSC are pluripotent cells that can differentiate along multiple cell lineages, including myogenic, osteogenic, neurogenic, and hematopoietic pathways [14, 25, 26] as like bone marrow stromal cells (BMSC). Culture-expanded hADSC (P3-P5) in this study expressed typical mesenchymal stem cell markers (CD29, CD44, CD90, CD105), but did not express hematopoietic markers (CD34, CD14, CD45) and endothelial markers (CD31, Flk-1). In contrast, recent reports showed that freshly isolated SVF and their early passage cells express CD34 up to 90% [24]. Our data showed that more than 90% of freshly expanded cells from adipose tissue (P0) expressed CD34, and that CD34 expression drastically

decreased during culture expansion. After 3 passages (about 6-7 population doubling) most cells did not express CD34. Because hADSC colonies that were derived from single CD34(+) cell did not show positive staining with CD34 antibody, decrease of CD34 expression in hADSC during culture expansion result from downregulation of CD34 transcription in hADSC, not from subset selection of CD34(-) cells.

It has been reported that CD34(+) cells obtained from SVF of adipose tissues have effective proangiogenic effect in vivo [21-24] reported that CD34(-) and Flk-1(+) cells in adipose tissues also have similar effect of CD34(+) cells. Our study showed that MSC derived from adipose lineage cells (CD34(-), CD31(-), Flk-1(-) hADSC) have the potential to differentiate into an endothelial cell phenotype in vitro, and that the transplantation of hADSC induce a marked increase in the blood flow as well as in the capillary density in the ischemic hindlimb of nude mice. We also showed that the effect of culture-expanded CD34(-) hADSC on ischemic hindlimb was similar with that of CD34(+) cell in fresh SVF of adipose tissue. These results support that culture-expanded MSC isolated from human adipose tissues can be used for the treatment of ischemic tissues.

In this study, hADSC can be differentiated into endothelial cells. Contamination of circulating endothelial progenitor cells, which have been shown to mainly derive from monocyte/macrophages [33, 34] or resident microvascular endothelial cells, may explain endothelial differentiation of hADSC. A monocyte/macrophage origin can here be excluded because hADSC are clearly negative for CD45 and CD14, panhematopoietic and macrophage antigens, respectively. We can also rule out that hADSC-induced vessel growth might be related to the proangiogenic effect of resident microvascular endothelial cells, because hADSC do not express any endothelial marker. Furthermore, the high proportion of differentiated cells during endothelial differentiation supports transdifferentiation of hADSC into endothelial cells. The ability of hADSC to differentiate into vascular cells is very consistent with recent reports, which suggest that adipose tissue vessels are in a relatively immature state compared with other organs that display a lower plasticity [18]. BMSC have been demonstrated to differentiate into smooth muscle and endothelial cell lineages [12, 35, 36], and thus may contribute cells directly to new or remodeling vessels. Nonetheless, the importance of the mechanism is still controversial, because the actual magnitude of incorporation of hADSC into vascular structures was quite low (less than 1%). Low incorporation rate of

transplanted cells observed in this study has been also reported in other studies including adipose-derived cells and BMSC transplantation in spite of impressive improvement in perfusion [37, 38]. These data suggest that other mechanisms apart from cell incorporation may contribute to collateral remodeling observed after hADSC transplantation.

Recent studies have demonstrated that the angiogenic potential of MSC is attributed not only to their differentiation into vascular endothelial cells but also to their ability to produce various angiogenic factors, including VEGF and bFGF [39]. The secretion of angiogenic and antiapoptotic factors by human adipose stromal cells has been reported [23]. In this study angiogenesis cytokine array experiments showed that the conditioned media obtained from hADSC contain multiple angiogenic cytokines including ELR(+) CXC chemokines (IL-8, ENA-78, GRO), all members of which are involved in angiogenesis [40]. The addition of the hADSC-CM to the endothelial culture media stimulated endothelial proliferation, and protected apoptotic cell death in vitro. These results therefore demonstrate that hADSC can augment collateral remodeling and appear to accomplish this mainly through paracrine pathways.

Local delivery of hADSC may also cause circulating stem/progenitor cells to home to the region of injury and contribute to healing. In the present study we documented hADSC release of several stem/progenitor cell chemokines, including VEGF and MCP-1. Previous studies have documented that MSC release other stem/progenitor cell chemokines, including hepatocyte growth factor and stem cell-derived factor [41]. Therefore, although we did not examine the possibility directly, it is highly likely that the collateral enhancing effects of cell therapy are mediated through multiple pathways, including paracrine effects on local vascular cells and chemoattractant effects leading to homing of circulating stem and or progenitor cells.

In most studies, stem cells were transplanted into ischemic hindlimb at the same day or the following day after ischemia. However, this protocol is not relevant to most clinical cases. Most patients suffering from peripheral vascular diseases have long-term medical history. It is impossible to acquire autologous cells enough for transplantation by 24 hours after vessel occlusion. In this study, the transplantation of hADSC at the 7th day after ischemia also showed proangiogenic effect in nude mice. The extent of blood flow recovery by hADSC transplantation at the 7th day after ischemia was better than that by their transplantation at the following day after

ischemia, which may be related to better survival of transplanted cells by subsidence of inflammatory responses in ischemic tissues.

Liposuction for harvesting adipose tissue from which to obtain hADSC is technically straightforward, safe, causes relatively little discomfort, and is free of ethical drawbacks. Furthermore, the effectiveness of culture-expanded hADSC transplantation even in 7 day ischemia shown in this study provides clear advantages for their clinical application in ischemic peripheral vasculopathy,

because cell numbers to be required for transplantation can be obtained from small amount of tissues.

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