Novel Autologous Cell Therapy in Ischemic Limb Disease Through Growth Factor Secretion by Cultured Adipose Tissue–Derived Stromal Cells

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Objective—The delivery of autologous progenitor cells into ischemic tissue of patients is emerging as a novel therapeutic option. Here, we report the potential impact of cultured adipose tissue–derived cells (ADSC) on angiogenic cell therapy.

Method and Results—ADSC were isolated from C57Bl/6 mouse inguinal adipose tissue and showed high expression of Sca1 and CD44, but not c-kit, Lin, CD34, CD45, CD11b, and CD31, compatible with that of mesenchymal stem cells from bone marrow. In coculture conditions with ADSC and human aortic endothelial cells (ECs) under treatment with growth factors, ADSC significantly increased EC viability, migration and tube formation mainly through secretion of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). At 4 weeks after transplantation of ADSC into the ischemic mouse hindlimb, the angiogenic scores were improved in the ADSC-treated group, which were evaluated with blood flow by laser Doppler imaging (LDI) and capillary density by immunostaining with anti-CD31 antibody. However, injected ADSC did not correspond to CD31, von Willebrand factor, and α-smooth muscle actin-positive cells in ischemic tissue.

Conclusion—These adipose tissue–derived cells demonstrated potential as angiogenic cell therapy for ischemic disease, which appears to be mainly achieved by their ability to secrete angiogenic growth factors. (Arterioscler Thromb Vasc Biol. 2005;25:2542-2547.)

Key words: adipose tissue ■ angiogenesis ■ growth factors ■ HGF ■ VEGF

Therapeutic angiogenesis, a strategy to treat tissue ischemia by promoting the proliferation of collateral vessels, has emerged as one of the most promising therapies developed to date.1-2 Therapeutic potential of various angiogenic molecules has been reported in animal models or humans with ischemic disease.3-4 Recently, autologous transplantation of bone marrow (BM) stromal cells or endothelial cell (EC) progenitors has been shown to enhance angiogenesis or peripheral blood flow, and these cells were incorporated into sites of angiogenesis after tissue ischemia in the limb, retina, and myocardium.5-7 However, it has also been suggested that bone marrow mononuclear cells contain various characteristics of stem cells for mesenchymal tissues8 and secrete a broad spectrum of angiogenic or antiangiogenic cytokines, such as IL-1β, which would play a role in the process of angiogenesis.9

Adipose tissue, like bone marrow, is derived from the embryonic mesenchyme and contains stroma that is easily isolated,10 which was classically used to investigate preadipocyte differentiation into mature adipocytes. However, this fraction was also reported to be a very convenient and nonrestrictive source of pluripotent cells such as hematopoietic progenitors and spare mesodermal stem cells able to differentiate into osteogenic, chondrogenic, myogenic, and neurogenic lineages.11,12 Moreover, in rodents, the presence of hematopoietic stem cells in adipose tissue–derived stromal cells (ADSCs) has been suggested, and the presence of a cell population expressing the stem cell marker CD34 has been shown in ADSC from human adipose tissue.13 These cells could differentiate into endothelial cells and participate in vessel formation, and interestingly, administration of adipose tissue–derived cells could potentially affect revascularization to a similar degree to BM-MNC administration.14 Of importance, ADSC secrete multiple angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), at levels that are bioactive.15,16 However, how to use the advantage of “cultured ADSC” for angiogenic therapy still needs to be determined.

In this study, we developed cultured ADSC from mouse inguinal fat, which showed fairly homogeneous characteristics compatible with mesenchymal stem cells. ADSC can...
differentiate into endothelial cells and secrete angiogenic growth factors that strongly induce endothelial cell growth, migration, and tube formation. Transplantation of these cultured ADSC to ischemic limbs accelerated angiogenesis mainly caused by secretion of growth factors, rather than participation in vessel formation by differentiation.

Materials and Methods

Cell Culture
ADSCs were isolated from adipose tissue according to the method of Bjornorp et al with minor modification.17 Human aortic endothelial cells and aortic smooth muscle cells (passage 3) were obtained from Clonetics Corp (San Diego, Calif), and cultured in modified MCDB131 medium (EGM2-MV; Clonetics Corp) or smooth muscle medium supplemented with 5% fetal bovine serum and smooth muscle growth supplement (Clonetics Corp) in the standard fashion.18 Detail information is described in Supplement (http://atvb.ahajournals.org).

Fluorescence-Activated Cell Sorting and Immunostaining
A total of 2×105 cells were resuspended in 200 μL phosphate-buffered saline containing 3% bovine serum albumin and incubated for 30 minutes with fluorescence-labeled antibodies against Ly-6A/E (Sca1)-PE (phycoerythrin), CD117 (c-kit)-fluorescein isothiocyanate (FITC) (FTIC), CD31-PE, CD34-PE, CD45-PE, CD11b-PE, CD44-PE, or the respective isotype control, which were obtained from BD Biosciences (Franklin Lakes, NJ). Cells were also incubated with biotin-conjugated mouse lineage panel antibodies against CD3e, CD31, CD34, or CD45 (Figure 1A). This suggests that ADSC include a large population of mesenchymal stem cells, but no hematopoietic stem cells.23 Of importance, these characteristics of ADSC surface markers were conserved after several passages of cell expansion. These cells look like fibroblasts and can differentiate into not only adipocytes but also chondrogenic and osteogenic lineages in vitro as previous described.17 To examine the pluripotency of ADSC, we tried to induce them to differentiate toward endothelial cells. Because murine embryonic stem cells or human endothelial progenitor cells can differentiate into endothelial cells with growth factor-rich medium in collagen-coated dishes,24 we seeded ADSC into collagen I-coated dishes with DMEM or growth factor-rich EGM2-MV medium for 5 days. Although PECAM-positive or VE-cadherin positive cells were not observed in ADSC cultured in EBM-2 medium, we found a few PECAM-positive or VE-cadherin-positive cells in growth factor-rich medium, which showed a spindle shape similar to endothelial progenitor cells from bone marrow (Figure 1, available online at http://atvb.ahajournals.org). To further confirm the differentiation of ADSC into EC, we also followed Flk-1, Tie-2, and PECAM gene expression by reverse-transcription PCR. After differentiation of murine ADSC in growth factor-rich EGM medium, we collected the PECAM-positive endothelial cells from ADSC using mouse specific anti-PECAM antibody-attached magnet beads and extracted mRNA from them as differentiated EC. As shown in Figure 1B, we confirmed the mRNA expression of Flk-1, Tie-2, and PECAM only in differentiated EC.

Results

Pluripotency of ADSCs
The inguinal fat pads from each mouse yielded approximately 1×106 nucleated cells. Freshly isolated ADSC showed adherence and expansion in culture. After second passage of cell expansion, FACS analysis of cultured ADSC indicated that Sca1 and CD44 antigen were expressed, but not c-kit, Lin, CD11b, CD31, CD34, or CD45 (Figure 1A). This suggests that ADSC include a large population of mesenchymal stem cells, but no hematopoietic stem cells.23 Of importance, these characteristics of ADSC surface markers were conserved after several passages of cell expansion. These cells look like fibroblasts and can differentiate into not only adipocytes but also chondrogenic and osteogenic lineages in vitro as previous described.17 To examine the pluripotency of ADSC, we tried to induce them to differentiate toward endothelial cells. Because murine embryonic stem cells or human endothelial progenitor cells can differentiate into endothelial cells with growth factor-rich medium in collagen-coated dishes,24 we seeded ADSC into collagen I-coated dishes with DMEM or growth factor-rich EGM2-MV medium for 5 days. Although PECAM-positive or VE-cadherin positive cells were not observed in ADSC cultured in EBM-2 medium, we found a few PECAM-positive or VE-cadherin-positive cells in growth factor-rich medium, which showed a spindle shape similar to endothelial progenitor cells from bone marrow (Figure 1, available online at http://atvb.ahajournals.org). To further confirm the differentiation of ADSC into EC, we also followed Flk-1, Tie-2, and PECAM gene expression by reverse-transcription PCR. After differentiation of murine ADSC in growth factor-rich EGM medium, we collected the PECAM-positive endothelial cells from ADSC using mouse specific anti-PECAM antibody-attached magnet beads and extracted mRNA from them as differentiated EC. As shown in Figure 1B, we confirmed the mRNA expression of Flk-1, Tie-2, and PECAM only in differentiated EC.

Effects of ADSC on EC Viability, Migration, and Tube Formation
Increasing numbers of reports highlight the possibility that adipogenesis and neovascularization are reciprocally regulated and tightly linked in rodents.25,26 Because the proliferation and migration of endothelial cells are important aspects of angiogenesis, we examined the effect of ADSC-conditioned medium in a coculture system of human aortic endothelial cells. Cell viability of endothelial cells in the conditioned medium from ADSC was significantly higher.
factors, such as VEGF and HGF, at levels that are bioactive. It is known that ADSC secrete multiple angiogenic growth factors (length, area, joint, path) compared with the conditioned medium also showed a significant increase in several parameters (angiogenesis-related cytokines from ADSC) in EGM2-MV medium (Figure 2C).

Figure 1, A, Murine ADSC were stained with anti-Scal, c-kit, Lin, CD45, CD34, CD44, CD11b, or Flk1 antibody and analyzed by flow cytometry. Red lines show cells stained with anti-Scal-PE, c-kit-FITC, CD34-PE, CD45-PE, CD11b-PE, CD44-PE, CD31-PE, or Lin-biotin-PE antibody, respectively, and green area shows the respective isotype control for each antibody. The means/SEM of positive signals were also shown for each antibody. B, Typical example of RT-PCR analysis of endothelial cell marker expression. RNA was extracted from ADSC or differentiated ADSC into EC, which were cultured in growth factor-rich EGM2-MV medium, and were collected with murine PECAM-positive antibody attached Dynabeads. Expression of Flk-1, Tie-2, and PECAM was examined as endothelial cell markers. G3PDH was used for normalization of total mRNA.

Angiogenic Effect of ADSC in Mice Ischemic Hindlimb Model
To determine whether transplantation of ADSC could induce an angiogenic effect, we evaluated the angiogenic effect of injected murine ADSC in the mouse ischemic limb. At 10 days after unilateral ligation of femoral artery of the mouse hindlimb, we performed LDI and separated the mice into three groups: injection of PBS or 1 x 10^6 ADSC cultured with growth factor-rich or withdrawal EGM2-MV medium. Figure 4A shows representative LDI images of hindlimb blood flow at 14 and 28 days after transplantation. Although serial LDI examination disclosed natural recovery of hindlimb blood flow in the control group, transplantation of ADSC resulted in more rapid recovery of the ratio of ischemic/normal blood flow. Interestingly, transplantation of ADSC in growth factor-rich EGM2-MV medium further improved it.

To investigate the degree of angiogenesis at the microcirculation level, we measured capillary density in histological sections harvested from the ischemic tissues. The representative photomicrographs of tissue immunostained with anti-CD31 antibody were shown in Figure III (available online at http://atvb.ahajournals.org). Quantitative analysis revealed that the capillary density was significantly increased by ADSC in growth factor-rich EGM2-MV medium, and to a lesser extent by...
ADSC in growth factor-withdrawal EGM2-MV medium, compared with the control group (Figure 4B).

We further performed the comparative study with bone marrow nuclear cells (BMN) and HGF gene therapy that had been already performed in human clinical trial for peripheral arterial diseases. In the analysis of blood flow by LDI at 28 days after each treatment, there was no significant difference between three groups (Figure III).

### Involvement of ADSC in Vasculogenesis

To confirm whether ADSC are directly involved in the development of vasculogenesis in the ischemic limb, we isolated ADSC from mice engineered to constitutively express GFP and injected ADSC cultured in EGM2-MV medium into the mouse ischemic limb as described. At 3, 14, or 28 days after injection, we isolated muscle and observed GFP-positive cells. As shown in Figure 5, we identified a few clusters of GFP-positive cells in the ischemic area, which may have been the injected area, at 3 days after cell transplantation, and fewer GFP-positive cells at 14 days, and almost no GFP-positive cells by immunostaining with anti-GFP antibody at 28 days, which did not correspond to the distribution of CD31, von Willebrand factor and -smooth muscle actin-positive cells. It suggests that ADSC might be not directly involved in the development of vasculogenesis in this model.

### Discussion

The present study demonstrated that transplantation of ADSC induced angiogenesis accompanied by an increase of blood flow and capillary density in the ischemic limb. Despite the promising potential for regenerative applications, the fundamental scarcity of endothelial progenitor cell populations in the hematopoietic system constitutes an important...
limitation of primary endothelial progenitor cell transplantation. It was reported that ex vivo expansion of endothelial progenitor cells cultured from the peripheral blood of healthy human volunteers yielded \(5.0 \times 10^6\) cells per 100 mL of blood, whereas heterologous transplantation requires \(0.5\) to \(2.0 \times 10^4\) human endothelial progenitor cells per gram of body weight (of the recipient mouse) to achieve satisfactory reperfusion of the ischemic hindlimb.\(^{27}\) Actually, the amount of autologous BM blood aspirated for therapeutic neovascularization was approximately 500 mL per person (ie, 0.1% of body weight.)\(^{7}\) This suggests a practical limitation of endothelial progenitor cell transplantation; namely, the volume of blood required to extract an adequate number of endothelial progenitor cells for autologous transplantation. The main benefit of our ADSC is that they can be easily harvested from patients by a simple, minimally invasive method and also easily cultured (\(1 \times 10^7\) cells from one mouse and 20 mL liposapsulation from a human). Moreover, cultured ADSC can be expanded more rapidly (>10 times within 1 week), and long-term cultured cells after some passages still retain their mesenchymal pluripotency, with expression of Sca-1 and CD44. This suggests that ADSC could be a good candidate as a novel source of cell therapy in cardiovascular disease.

Recently, therapeutic endothelial stem cell transplantation was suggested to be a promising approach to restore tissue vascularization after ischemic events.\(^6\) However, the true in vivo differentiation capacity of adult BM stem and progenitor cells and their possible contribution to nonhematopoietic cells and tissues including endothelial cells remain controversial.\(^{28,29}\) We speculate that the injection of cells are not mimicking strictly the natural course of the cells infused or the cultured stem cells under special conditions can change the properties of these cells in terms of their capacity to incorporate into target tissue. In addition, we have to observe carefully whether the survive cells would be a “differentiation” or “fusion.” A very recent report suggested that adult BM-derived cells participate in angiogenesis, and a small subpopulation of them differentiate into vascular mural peri-

**Figure 4.** Effect of ADSC injection in mouse ischemic limb model. A, Representative image of peripheral blood flow analyzed by LDI at 2 or 4 weeks after injection. Color-coded images represent blood flow distribution; low or no perfusion is displayed as blue, whereas highest perfusion is displayed as red. Quantitative analysis of blood flow in hindlimbs is expressed as perfusion ratio of ischemic hindlimb to untreated opposite limb. B, Capillary density in cross sections of ischemic tissue immunostained with anti-CD31 (PECAM) antibody. Control indicates phosphate-buffered saline injection; ADSC (EBM), indicates ADSC maintained in EBM-2 medium (without growth factor); ADSC (EGM), ADSC maintained in EGM2-MV medium (with growth factor). Each group contains 7 or 8 animals. \(^*P<0.01\) vs Control.

**Figure 5.** A, GFP-expressing ADSC in cross section of ischemic tissue visualized under a microscope. Left panel indicates 3 days after GFP-expressing ADSC injection, and right panel indicates 14 days after GFP-expressing ADSC injection. Bar=100 \(\mu\text{m}\). B, HE staining (left) and immunostaining with anti-PECAM (center) and anti-GFP (right) antibody in cross sections of ischemic tissue at 28 days after GFP-expressing ADSC injection. Upper panel shows low magnification and lower panel shows high magnification. Bar=100 \(\mu\text{m}\). C, The immunostaining with anti-vWF (von Willebrand factor) or \(\alpha\)-SMA (smooth muscle actin) (left) and anti-GFP (right) antibody in cross sections of ischemic tissue at 28 days after GFP-expressing ADSC injection. Bar=100 \(\mu\text{m}\).
endothelial cells that are morphologically indistinguishable from pericytes. BM contains nonhematopoietic stromal cells, which comprise immature mesenchymal stem cells, EPC, fibroblasts, osteoblasts, EC, and adipocytes, and infiltrating inflammatory leukocytes release angiogenic cytokines including VEGF. Interestingly, treatment with anti-VEGF monoclonal antibody completely abolished BM-induced neovascularization. This suggests that stem cell transplantation is also “cell-based cytokine therapy.” Of importance, in this study we used media containing growth factors to induce the direction of differentiation into vascular cells, to avoid differentiation into unexpected cells, and also activate the secretion of angiogenic growth factors leading to “cell-based cytokine therapy.” Although in this study our ADSC-mediated cell therapy did not show further angiogenic effect in the comparison with BM cell or HGF gene therapy, it would be a study limitation to conclude their effectiveness in this ischemic hindlimb model. Our cultured ADSC can provide stem cells by FACS analysis and are easily available for autologous cell therapy, which would allow feasible implementation of “stem cell-based functional gene therapy” in the future.

Increasing numbers of reports highlight the possibility that adipogenesis and neovascularization are reciprocally regulated and tightly linked in rodents. Our results indicate that ADSC represent an easily accessible cell source with potential effects that can be used for therapeutic angiogenesis through growth factor secretion, so-called adipocytokine.

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