Implantation of Adipose-Derived Regenerative Cells Enhances Ischemia-Induced Angiogenesis

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Objective—Therapeutic angiogenesis using autologous stem/progenitor cells represents a novel strategy for severe ischemic diseases. Recent reports indicated that adipose tissues could supply adipose-derived regenerative cells (ADRCs). Accordingly, we examined whether implantation of ADRCs would augment ischemia-induced angiogenesis.

Method and Results—Adipose tissue was obtained from C57BL/6J mice, and ADRCs were isolated using standard methods. ADRCs expressed stromal cell–derived factor 1 (SDF-1) mRNA and proteins. Hind limb ischemia was induced and culture-expanded ADRCs, PBS, or mature adipocytes (MAs) as control cells were injected into the ischemic muscles. At 3 weeks, the ADRC group had a greater laser Doppler blood perfusion index and a higher capillary density compared to the controls. Implantation of ADRCs increased circulating endothelial progenitor cells (EPCs). SDF-1 mRNA abundance at ischemic tissues and serum SDF-1 levels were greater in the ADRC group than in the control group. Finally, intraperitoneal injection of an anti–SDF-1 neutralizing antibody reduced the number of circulating EPCs and therapeutic efficacies of ADRCs.

Conclusions—Adipose tissue would be a valuable source for cell-based therapeutic angiogenesis. Moreover, chemokine SDF-1 may play a pivotal role in the ADRCs-mediated angiogenesis at least in part by facilitating mobilization of EPCs. ***(Arterioscler Thromb Vasc Biol.* 2009;29:61-66.)**

Key Words: angiogenesis ■ adipose-derived regenerative cells ■ progenitor cells ■ chemokine

When tissue is exposed to severe ischemia, new blood vessels develop into the ischemic foci to prevent tissue necrosis. Because circulating endothelial progenitor cells (EPCs) have been shown to participate in postnatal neovascularization after mobilization from the bone marrow (BM),[1,2] we have performed basic and clinical studies related to therapeutic angiogenesis using EPCs or BM cells.[3–5] We have performed therapeutic angiogenesis using autologous BM mononuclear cell (BM-MNCs) implantation into the ischemic muscles in patients with critical limb ischemia (TACT).[6–8] Although the safety and efficiency of the TACT protocol have been established, we recently reported that patients with very severe peripheral artery occlusive disease had poor responses to the TACT procedure.[7] Moreover, recent data indicated that patients with severe obstructive vascular disease or multiple coronary risk factors had diminished functions of EPCs and poor responses to angiogenic cell therapy.[9–12] Thus, alternative source of stem/progenitor cells for therapeutic angiogenesis has been searched extensively.

Recently, several investigators have reported that adipose tissues contain multipotent mesenchymal stem cells termed adipose-derived regenerative cells (ADRCs), which have an ability to regenerate damaged tissues.[13–15] However, little is known as to how implantation of ADRCs would induce angiogenesis in ischemic tissues. It has been known that ADRCs secrete multiple angiogenic growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).[13,14] Such growth factors would mobilize EPCs from the BM into peripheral blood (PB) and finally to ischemic tissues. However, there is limited evidence regarding the effects of in vivo implantation of ADRCs on EPC kinetics during ischemia-induced angiogenesis.

Accordingly, we examined whether implantation of ADRCs might augment angiogenesis, collateral vessel formation, and mobilization of EPCs in a mouse model of hind limb ischemia.

Methods

Isolation of Mouse ADRCs

All protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. ADRCs were isolated from inguinal fat pads of C57BL/6J mice (n=32) and of GFP-transgenic mice with C57BL/6J background (n=3; kindly provided by Dr M. Okabe at Osaka University) as described

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previously[15–17] Methods in detail are described in the supplemental materials (available online at http://atvb.ahajournals.org).

Adipocyte Differentiation Assay

Adipogenic differentiation of ADRCs was introduced as previously described (please see supplemental materials).[15] Adipogenic differentiation was confirmed by Oil Red O staining. To examine whether ADRCs can give rise to EPCs or mature endothelial cells (ECs), ADRCs were cultured in EBM-2 (endothelial cell basal medium; Clonetics) supplemented with EGM-2 MV. At day 7, attaching cells were stained with incorporation of 1,1′-dioctadecyl-1 to 3,3,3′,3′-tetramethylindo-carbocyanine perchlorate-labeled acetylated LDL (DiI-acLDL, Biomedical Technology Inc) and binding of fluorescein isothiocyanate (FITC)-labeled *Bandeiraea simplicifolia* lectin 1 (FITC-BS-1 lectine, Vector Laboratories), and antiplatelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) monoclonal antibody (mAb) (Becton Dickinson), and anti α-smooth muscle actin (α-SMA) mAb (Abcam).[1,18] After immunofluorescence staining, nuclei were stained with DAPI (Invitrogen).

Characterization of ADRCs by Flow Cytometry

A total of 5×10^5 cells were incubated for 30 minutes at 4°C with mAbs against Ly-6A/E (*Sca*I), CD31, CD34, c-kit, flk-1, and Lin (BD Biosciences). To characterize the phenotypes of ADRCs, fluorescence-activated-cell sorter (FACS) analysis was performed using the FACS Caliber instrument (Becton Dickinson) and Cell Quest software (BD Biosciences).[18]

Real-Time Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA was isolated from cultured ADRCs and differentiated MAs using TRIzol Reagent (Invitrogen Life Technologies). Total RNA from the frozen tissues with liquid nitrogen (LN_2) obtained at days 0, 3, and 7 after hind limb ischemia was extracted using FastPrep System (BIO 101). Real-time RT-PCR analysis of the VEGF, SDF-1, and GAPDH mRNAs was performed using 2 μg total RNA on Mx3000P Real-Time PCR System (Stratagene) using SYBR Green I as a double-stranded DNA-specific dye according to manufacturer's instruction (Applied Biosystem).[19] mRNA levels were expressed relative to the levels of GAPDH. Further information is described in the supplemental materials.

ELISA

Conditioned medium were obtained from cultured ADRCs and MAs at 72 hours after final change of fresh DMEM/10%FBS. Concentrations of SDF-1α and VEGF proteins in the media were determined by ELISA (mouse CXCL12/SDF-1α Quantikine ELISA kit and mouse VEGF ELISA kit, R&D Systems) according to manufacturer's instruction. Plasma levels of SDF-1α and VEGF at days 0, 3, and 7 after hind limb ischemia were also measured.

Mouse Model of Unilateral Hind Limb Ischemia

Unilateral hind limb ischemia was induced in male C57BL/6J mice (6 to 10 weeks old, n=32; Nihon Crea) as described previously.[20] No mice died during the experimentation. Mice were randomly divided into 3 groups. The control group (n=8) received phosphate-buffered saline (PBS). The ADRC group (n=8) received ADRCs (1×10^6 cells per animal), and the MA group (n=6) received mature adipocytes (MA) (1×10^6 cells per animal) implanted at 3 predetermined points of the ischemic muscles at postoperative day 1. After treatment, angiogenesis and collateral vessel formation in the ischemic tissues were analyzed as described previously.[20] In addition, we examined whether SDF-1 is necessary for augmentation of angiogenesis in ischemic tissues by ADRCs implantation. Some mice received intraperitoneal anti–SDF-1 neutralizing mAb (50 μg; R&D system) 3 times per week up to 14 days after surgery. As a control, nonspecific rat IgG was administered in a similar manner. Further information is described in the on-line supplement. Please see the supplemental materials.

To investigate whether implanted ADRCs could survive, differentiate into ECs or pericytes, and secrete angiogenic cytokines in ischemic tissues, some mice received GFP-transgenic mice-derived ADRCs. This subgroup of mice was euthanized at postoperative days 3 and 28 (n=5 for each). At day 28, 2 mice received 50 μg of Rhodamine-labeled BS-1 lectin (Rhodamine-BS-1 lectin, Vector Laboratories) intravenously 30 minutes before sacrifice to visualize vessels. Frozen sections were further stained with anti-CD31, anti-CD140b (Thermo Scientific), anti–SDF-1 mAbs (BioVision), or anti-VEGF mAb (Thermo Scientific), and the nuclei were stained with DAPI. Adjacent sections were stained with hematoxylin-eosin (H&E).

EPC Kinetics Assay by Cell Culture and Flow Cytometry

PB-MNCs and BM-MNCs obtained before limb ischemia surgery and at days 3 and 7 after limb ischemia were isolated and cultured with EGM2-MV as described previously.[21,22] After 4 days of culture, EPCs were identified by uptake of DiI-Ac-LDL and binding of FITC-BS-1 lectine.[23]

EPC populations in BM-MNCs and PB-MNCs were further analyzed using FACS with FITC-labeled anti–Sca-1 mAb and PE-labeled antiflk-1 mAb (BD Bioscience).[22] Isotype-matched IgGs were used as negative controls. Immnuofluorescence-labeled cells were analyzed by FACS and Cell Quest Software counting 10 000 events per sample.[24] In addition, we examined whether EPC kinetics is affected by blocking SDF-1 after ADRC implantation in ischemic tissues. Some mice received intraperitoneal anti–SDF-1 neutralizing mAb (50 μg; R&D System) or nonspecific rat IgG as described above.

Statistics

Data are expressed as mean±SEM. Statistical significance was evaluated using unpaired Student *t* test for comparison between 2 means and ANOVA for comparison among 3 groups. Values of $P<0.05$ denoted statistical significance.

Results

Characterization of Cultured ADRCs

ADRCs looked like fibroblast-like attaching cells and were culture expandable (supplemental Figure IAa and IAb). These cells could differentiate into mature adipocytes (MA) under adipogenic differentiation medium with high glucose concentration. Differentiation of ADRCs into MAs was confirmed by microscopic observation of intracellular lipid droplets and positive staining with Oil Red O (supplemental Figure IAc and IAd).

Immunocytochemistry revealed that these adherent cells were positive for α-SMA (supplemental Figure IAe), but cells cultured in endothelial differentiation medium (EGM2-MV) were negative for CD31 (supplemental Figure IAf), DiI-acLDL incorporation and FITC-BS1 lectin binding (supplemental Figure IAg), indicating that EGM2 endothelial culture condition could not lead ADRCs differentiate into endothelial lineage. Moreover, FACS analysis of culture-expanded ADRCs were positive for Sca-1 but not for CD31, CD34, c-kit, Lin, and flk-1, markers of differentiated cells (supplemental Figure IAh).

Angiogenic Cytokine Production by ADRCs

We next examined the expression of VEGF and SDF-1 mRNAs in cultured ADRCs and MAs by real-time RT-PCR. Abundance of VEGF mRNA of ADRCs was not significantly different from that of MAs. However, abundance of SDF-1 mRNA of ADRCs was significantly greater (2.7-fold, n=4,

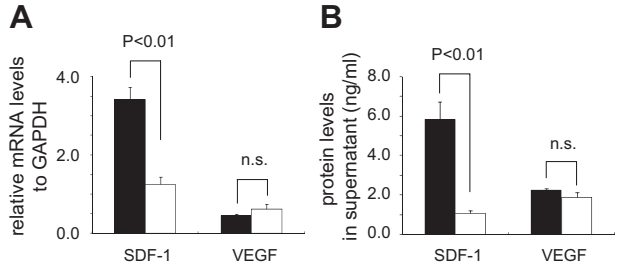

Figure 1. A, Adipogenic induction significantly reduced SDF-1 mRNA, but not VEGF mRNA assessed by quantitative RT-PCR. B, Secretion of SDF-1α and VEGF by cultured ADRCs significantly reduced by adipogenic induction. (ADRCs without adipogenic induction; black columns, ADRCs with adipogenic induction; white columns).

$P<0.01$) than that of MAs (Figure 1A). Then, to investigate whether ADRCs secrete SDF-1α and VEGF proteins, we performed ELISA assay in culture conditioned media. Although concentrations of VEGF in conditioned medium from ADRCs showed no significant difference compared to those of MAs, concentrations of SDF-1α of conditioned media from ADRCs was significantly greater (5.4-fold, n=4, $P<0.01$) than those of MAs (Figure 1B). These data were in accordance with those of mRNA expressions.

Augmentation of Ischemia-Induced Neovascularization by ADRC Implantation

We examined whether in vivo implantation of ADRCs could augment ischemia-induced angiogenesis using a mouse model of hind limb ischemia. Representative images of laser Doppler blood perfusion image (LDPI) system are shown in supplemental Figure IIA. A greater degree of blood perfusion was observed in the ischemic limb of ADRCs-implanted mice compared to control or MAs-implanted mice. Figure 2A shows summarized data of the ischemic/normal hind limb LDPI ratio. Although a marked recovery of blood perfusion was observed in the ADRCs-implanted group, the LDPI ratio remained low in the other 2 groups (*$P<0.05$, †$P<0.01$ versus control). Interestingly, blood flow recovery in mice of the MA-implanted group showed a weaker recovery of LDPI ratio compared to saline-injected control animals.

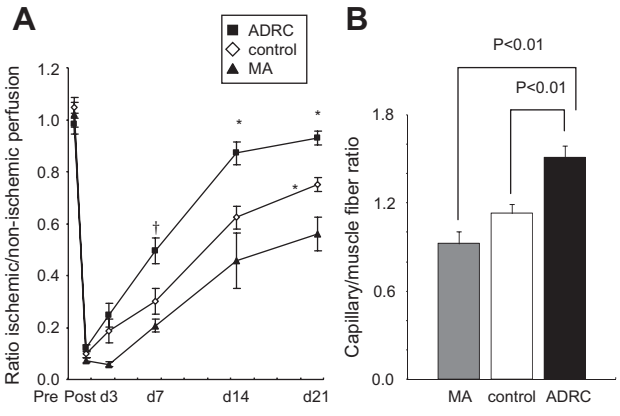

Figure 2. ADRCs implantation augmented ischemia-induced angiogenesis. A, Quantitative analysis of blood flow in the ischemic hind limb was expressed as the LDPI ratio of ischemic/non-ischemic hind limb. (*$P<0.05$, †$P<0.01$ vs control) B, Quantitative analysis of the capillary/muscle fiber ratio.

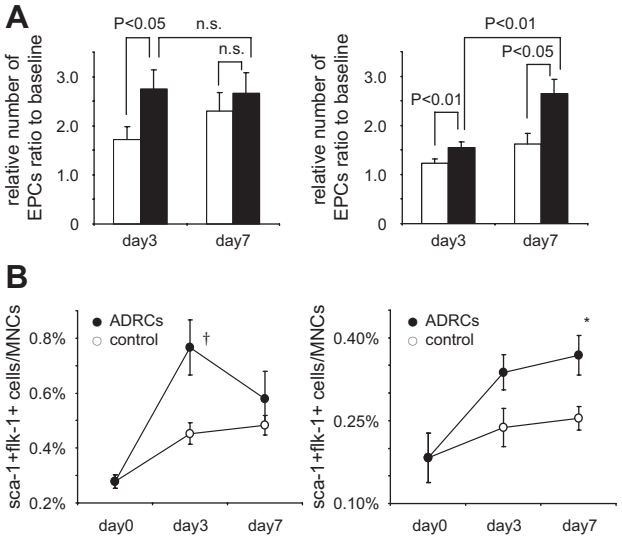

Figure 3. Quantitative analysis of EPCs in BM and circulating PB by culture assay (A: control group; white column, ADRCs group; black column) and flow cytometry (B, *$P<0.05$, †$P<0.01$). The number of EPCs mobilized from BM to PB increased by implantation of ADRCs.

We also measured the capillary density in histological sections harvested from the ischemic tissues. Representative photomicrographs are shown in supplemental Figure IIB. Quantitative analysis revealed that the capillary density at the ischemic skeletal muscles was significantly greater in the ADRCs group compared to the other 2 groups (n=5 for each group, *$P<0.05$, **$P<0.01$ versus control; Figure 2B). Again capillary density in the MA-implanted group was lower as compared to the control group (Figure 2B), consistent with the data of LDPI ratio.

Effects of ADRCs Implantation on Circulating and BM EPCs in the Hind Limb Ischemia Model

To assess EPC kinetics, we performed culture assay of PB- and BM-MNCs by double staining for DiI-acLDL incorporation and BS-1 lectin binding. Representative images of cultured EPCs are shown in supplemental Figure IIIA. A greater number of EPCs in the BM was observed at postoperative day 3 in the ADRCs group compared to the control group (2.8-fold increase in the ADRCs group versus 1.7-fold increase in the control group, n=6 for each group, $P<0.05$; Figure 3A left). In the PB, the number of EPCs increased at days 3 and 7 (1.6-fold increase at day 3 in the ADRCs group versus 1.2-fold increase at day 3 in the control group, $P<0.01$; and 2.7-fold increase at day 7 in the ADRCs group versus 1.6-fold increase at day 7 in the control group, n=6 to 8 for each group, $P<0.05$; Figure 3A right). These findings were corroborated with the data of FACS analysis of BM and PB samples collected at same time points, indicating that the number of Sca-1$^+$/Flk-1$^+$ cells was consistently greater in the ADRCs-implanted mice than in the PBS treated animals (Figure 3B). Representative images of FACS analysis are shown in supplemental Figure IIIB.

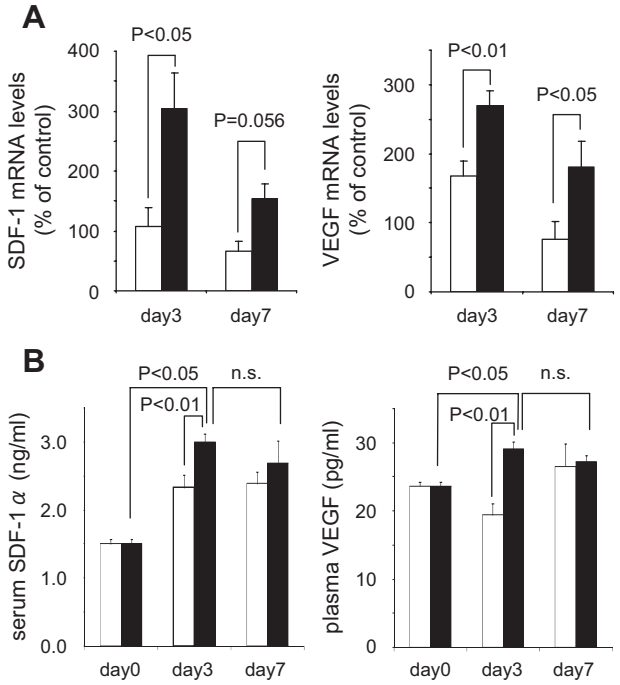

Figure 4. A, Expressions of SDF-1 (left) and VEGF (right) mRNA determined by real-time RT-PCR in the control (white columns) and ADRCs (black columns) groups expressing relative to baseline (day 0). B, Plasma concentrations of SDF-1α (left) and VEGF (right) measured in the control (white columns) and ADRCs (black columns) groups by ELISA.

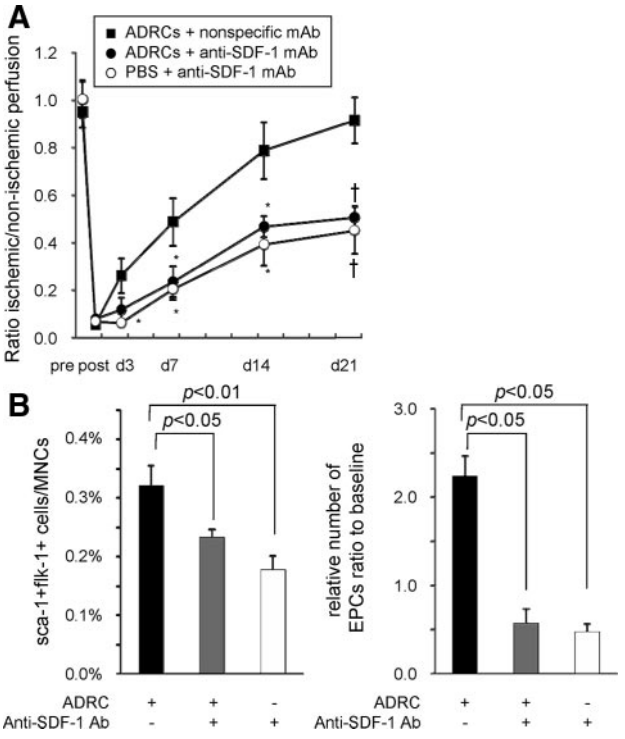

Figure 5. A, Quantitative analysis of the LDPI ratio after implantation of ADRCs with anti-SDF1mAb, implantation of ADRCs with control rat IgG and PBS with anti-SDF1mAb (*$P<0.05$, †$P<0.01$ vs control). B, Quantitative analysis of EPCs in circulating PB by culture assay (left) and FACS analysis (right).

Implanted ADRCs Augmented Secretion of Angiogenic Cytokines From Ischemic Tissues

We investigated whether ADRC implantation upregulates SDF-1 and VEGF mRNA expression in ischemic hind limb muscles. At postoperative day 3, SDF-1 mRNA abundance increased significantly (2.8-fold, n=7 to 8 for each group, $P<0.05$) in the ADRCs group compared to the control group (Figure 4A left). At days 3 and 7, VEGF mRNA abundance also increased significantly (1.6-fold, n=7 to 8 for each group, $P<0.01$) in the ADRCs group compared to the control group (Figure 4A right). Plasma SDF-1α (n=4 to 5 for each group, $P<0.01$) and VEGF (n=4 to 5 for each group, $P<0.01$) protein levels were also increased at day 3 in the ADRCs group compared to the control group (Figure 4B). Furthermore, to confirm whether implanted ADRCs secrete SDF-1 or VEGF proteins in the ischemic tissues, frozen sections from ischemic tissues of mice that received GFP-transgenic mice–derived ADRCs were stained with anti-SDF-1 or anti-VEGF mAbs. Some of these cells were positive for SDF-1 (supplemental Figure IVA) in the ischemic tissues at day 3. Although the most of these cells were negative for VEGF, VEGF was detected in the cytoplasm of skeletal myofibers nearby GFP positive cells in the ischemic muscles (supplemental Figure IVB). The number of cells stained positive for either SDF-1 or VEGF were significantly lower in the control group compared to the ADRCs group (supplemental Figure IVA and IVB).

Location of Implanted ADRCs at Chronic Phase

We examined whether in vivo implanted ADRCs could survive and differentiate into ECs at chronic phase using mice receiving GFP-transgenic mice-derived ADRCs. GFP positive cells were found in the ischemic area at post operative day 28 (supplemental Figure VA) and some of these cells seemed to be incorporated into Rhodamione-BS-1 lectin positive capillaries. (supplemental Figure VB and VC). Immunofluorescence staining revealed that some of the GFP-positive cells resided nearby vascular structures and capillaries stained with CD31 (supplemental Figure VD), and some of these cells were stained with anti-CD31 (supplemental Figure VE). In addition, these cells were positive for CD 140b, a pericyte maker (supplemental Figure VF). These results indicated that implanted ADRCs might contribute at least in part to vascular formation as pericytes in chronic phase. Furthermore, we could not detect formation of any tumors in transplanted animals until at least day 60 (n=3, data not shown).

SDF-1α Is Required for ADRCs Implantation-Induced Neovascularization

Intraperitoneal injection of an anti–SDF-1 neutralizing mAb significantly suppressed the angiogenesis mediated by ADRC implantation to the level equal to animals without ADRCs. Representative images of LDPI are shown in supplemental Figure VA. The LDPI ratio revealed that the blood flow recovery was significantly suppressed with the anti–SDF-1 mAb treatment (n=4 to 5, *$P<0.05$, †$P<0.01$ versus control; Figure 5A). Furthermore, the anti-SDF-1 mAb significantly reduced numbers of circulating EPCs at postoperative day 7 as assessed by culture assay and FACS analysis (n=5 to 7 for each group, *$P<0.05$, †$P<0.01$ versus control; Figure 5B).

Discussion

Major findings in the present study are as follows: (1) Cultured ADRCs expressed mesenchymal markers but not endothelial lineage markers in vitro. (2) ADRCs could differentiate into mature adipocytes (MAs), but these cells gave rise to neither EPCs nor mature ECs in vitro. (3) Direct local implantation of ADRCs but not MAs into ischemic hind limb muscles significantly augmented neovascularization. The angiogenic actions of ADRCs was markedly suppressed by an anti–SDF-1 neutralizing mAb treatment. Finally, (4) ADRC implantation increased SDF-1 release from ischemic tissues, which mobilized EPCs in vivo.

Adipose tissue mainly comprises 2 classes of cell population: one is mature adipocytes (MAs), and the other is stromal cells called "stromal vascular fraction" (SVF). Studies have shown that SVF contains multi-potent mesenchymal cells that differentiate into various lineage cells including fibroblasts, pericytes, osteoblasts, and myocytes.[16] We previously demonstrated that ADRCs could be isolated from small amount of human subcutaneous adipose tissue.[15] Although some studies reported that adipose-derived cells could differentiate into ECs or EPCs,[25,26] we could not confirm ADRCs to differentiate into endothelial lineages in the present study. The reason of this discrepancy is unknown, however Miranville and coworkers[26] used low-serum medium supplemented with VEGF and insulin-like growth factor-1 (IGF-1). The difference of such culture condition may affect maturation of ADRCs into ECs. Nevertheless, a recent study suggested that human ADRCs failed to differentiate into ECs even under culture with EGM-2,[27] being consistent with our findings. In addition, we could not confirm differentiation of ADRCs into ECs in vivo. Implanted ADRCs expressed no endothelial maker, but implanted ADRCs were positive for CD140b and colocalized with vessels like pericytes. These results suggest that ADRCs might not have capability to differentiate into ECs but smooth muscle lineage cells. Similarly, several recent studies showed that ADRCs differentiated into pericytes in vitro and in vivo.[28–30]

Nakagimi and coworkers and Sumi and coworkers reported that implanation of adipose-derived mesenchymal cells induced angiogenesis via secretion of angiogenic cytokines.[14,31] In the present study, we also found that implantation of ADRCs significantly augmented angiogenesis in a mouse model of hind limb ischemia. Our data indicate that implantation of ADRCs induced angiogenesis not by an endothelial differentiation but by chemokines such as SDF-1. Interestingly, we found that implantation of MAs into skeletal muscles even worsened angiogenesis compared to saline-injected control mice. We found that ADRCs expressed SDF-1, and the abundance of mRNA and protein were significantly greater in ADRCs than in MAs. In contrast, mRNA and protein abundance of VEGF did not differ between ADRCs and MAs. These results suggest that SDF-1 secreted from ADRCs may at least in part account for the difference in the angiogenic potency between ADRCs and MAs.[32] In addition, recent studies indicated that MAs release other adipocytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-6. These potentially deleterious inflammatory cytokines might have negatively affected angiogenesis by MA implantation observed in the present study.[28]

SDF-1 is a member of CXC chemokines originally isolated from murine BM stromal cells.[33] CXCR4 is the receptor for SDF-1 and is a coreceptor for HIV type 1 infection.[34] SDF-1/CXCR4 interaction regulates multiple physiological processes including embryonic development and organ homeostasis. Interestingly, SDF-1 is considered as one of the key regulators of EPCs trafficking from BM into PB.[19] Thus, SDF-1 has been shown to augment neovascularization by acceleration of EPC recruitment into ischemic tissues.[19,32] In addition, VEGF is one of powerful angiogenic cytokines that can mobilize EPCs from BM and inhibit EPC apoptosis.[18,23] In the mouse ischemic hind limb model, VEGF-A–mediated angiogenesis partly depends on the activation of the SDF-1–CXCR4 pathway.[35,36] Taken together, SDF-1 plays a pivotal role for the cell therapy–mediated angiogenesis. In fact, therapeutic efficacies and mobilization of EPCs of ADRCs implantation was markedly suppressed by i.p. injection of an anti–SDF-1 neutralizing mAb in the present study.

Regarding clinical trial of angiogenesis, we have reported the safety and efficiency of therapeutic angiogenesis using autologous BM-MNCs (TACT).[6] However, there are some patients who do not respond well to this procedure.[7] Zeiher and coworkers showed that EPC mobilization and functions were reduced in patients with ischemic cardiomyopathy compared to nonischemic subjects,[9,12] indicating that there may be a limited efficacy of the implantation of autologous BM cells for angiogenesis. Our current study indicates that autologous ADRCs are good alternatives to BM or circulating progenitor cells to induce angiogenesis.

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Disclosures

None.

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