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VEGF secretion by adipose tissue-derived regenerative cells is impaired under hyperglycemic conditions via glucose transporter activation and ROS increase

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ABSTRACT

Transplantation of cultured adipose-derived regenerative cells (ADRCs) into ischemic tissues promotes neovascularization and blood perfusion recovery. These effects are attenuated in diabetes patients. We examined the effects of hyperglycemia on the angiogenic capacity of ADRCs derived from Wistar rats both *in vivo* and *in vitro*. Cultured ADRCs were predominantly composed of CD90 positive cells; prevalence of CD90 positive cells was not affected by hyperglycemia. mRNA and protein levels of vascular endothelial growth factor (VEGF) were significantly decreased in ADRCs under hyperglycemic conditions independent of osmolarity, whereas mRNA levels of hepatocyte growth factor and fibroblast growth factor were unaffected. Since ADRCs express glucose transporter proteins GLUT1, 3 and 4, we examined the effects of the glucose transporter inhibitor phloretin on reactive oxygen species (ROS) and angiogenic factors. Phloretin decreased the glucose uptake rate, reduced ROS, and increased VEGF mRNA in ADRCs exposed to a hyperglycemic condition. *In vivo* transplantation of ADRCs cultured under hyperglycemic conditions into mouse ischemic limbs resulted in significantly decreased blood perfusion and capillary density in ischemic regions compared with transplantation of ADRCs cultured under normoglycemic conditions. These results suggest that hyperglycemia impaired VEGF production in ADRCs via an increase of ROS, impairing the angiogenic capacity of ADRCs transplanted into ischemic limbs.

Peripheral artery disease (PAD) is a progressive condition characterized by stenosis and occlusion of the leg arteries, developing from a symptomatic condition known as claudication to critical limb ischemia (15). Although several procedures such as percutaneous transluminal angioplasty and bypass surgery are available for treating PAD, their clinical outcome is not sufficient because of a high rate of restenosis or a low rate of long-term patency of the arteries (4).

Transplantation of stem or progenitor cells into ischemic tissues is emerging as a novel angiogenic therapy for the treatment of severe ischemic diseases, with excellent outcomes in clinical trials (13). Endothelial progenitor cells, which migrate to an

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ischemic site and differentiate into endothelial cells *in vivo*, are comprised in bone marrow-derived mononuclear cells (BM-MNCs) (2). However, the bone marrow contains a limited number of BM-MNCs; therefore, a large volume of the bone marrow must be collected for therapeutic angiogenesis. Thus, an alternative source of stem or progenitor cells is necessary. Recently, several investigators have reported that adipose tissue contains multipotent mesenchymal stem cells called "adipose-derived regenerative cells (ADRCs)" (23). Thus, the adipose tissue is an abundant source of regenerative cells available for autologous cell-transplantation; ADRCs can be easily obtained from adipose tissues with minimally invasive procedures. Furthermore, it has been reported that ADRCs secrete multiple angiogenic growth factors that promote neovascularization (19), and that transplantation of cultured ADRCs into an ischemic site improves blood perfusion and increases tissue capillary density (10). Instead of BM-MNCs, therefore, ADRCs may be useful for angiogenic cell therapy.

PAD is caused by arteriosclerosis in patients with life-style related diseases such as hypertension, hyperlipidemia and diabetes. Diabetes is one of the major risk factors causing PAD. Clinical studies demonstrated that angiogenesis by ADRCs was attenuated in diabetes; ADRCs obtained from patients with diabetes type 1 or 2 had reduced abilities of proliferation and VEGF secretion (11). Experimental studies showed that human ADRCs cultured under hyperglycemic conditions exhibited decreased proliferation potential (11). The functions of endothelial progenitors, muscle stem cells, and mesenchymal stem cells have been reported to be impaired when cultured in the presence of a high concentration of glucose (1). ADRCs isolated from diabetic rats and those cultured in medium containing high concentrations of glucose showed impaired proangiogenic functions (9). These results indicated that angiogenic properties of ADRCs might be impaired under hyperglycemic conditions. Yet, it remains unknown how angiogenic properties of ADRCs become impaired under such conditions.

In the present study, we studied the effects of hyperglycemic conditions on secretion of angiogenic factors in ADRCs *in vitro* and *in vivo*, and found that elevation of intracellular glucose attenuated VEGF secretion by ADRCs via generation of reactive oxygen species (ROS), impairing neovascularization in ischemic muscles transplanted with ADRCs.

MATERIALS AND METHODS

Isolation and culture of rat ADRCs. All protocols were approved by the Institutional Animal Care and Use Committee of Tottori University. Fresh ADRCs were isolated from the inguinal fat pad of Wistar rats (males, 8 weeks; CLEA Japan, Osaka, Japan) (18). Adipose tissues were minced and digested with 1 mg/mL type I collagenase (Wako, Osaka, Japan). After filtration through a 100 μm-pore filter (BD Biosciences, San Jose, CA, USA), ADRCs were recovered by centrifugation (2,200 rpm for 5 min) and removal of adipocytes. ADRCs were cultured in Dulbecco's Modified Eagle Medium (DMEM; SIGMA-Aldrich, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS) and penicillin-streptomycin-glutamine. Early passage cells were used in experiments. ADRCs were cultured under normoglycemic condition (with 5.5 mM glucose) for 3 passages and subsequently cultured in normoglycemic medium, high glucose (25 mM) medium, or normoglycemic but hyperosmotic medium with 19.5 mM mannitol for 2 weeks, and subjected to the further analyses.

Flow cytometric analysis. ADRCs were incubated with fluorescein conjugated antibodies against CD31 [fluorescein isothiocynate (FITC); AbD Serotec, Raleigh, NC, USA], CD45 [phycoeruthrin (PE); Bio Legend, San Diego, CA, USA], or CD90 [allophycocyanin (APC); Miltenyi Biotec, Auburn, CA, USA] for 10 min at 4°C in 0.5% FBS and 2 mM EDTA in phosphate-buffered saline (PBS). Cells were also incubated with fluorescein conjugated antibody against CD34 (FITC; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at 4°C in 0.5% FBS and 2 mM EDTA in PBS. Labeled cells were run on a BD FACS CantII flow cytometer (BD Biosciences). Data were analyzed using BD FACS Diva software (BD Biosciences).

Real-time reverse transcriptase-polymerase chain reaction analysis (RT-PCR). Extraction of total RNA from ADRCs was performed using RNeasy Mini kit (QIAGEN Inc., Valencia, CA, USA). Total RNA was extracted from frozen adductor skeletal muscle 3 days after surgery with or without transplanted ADRCs using RNeasy fibrous tissue kit (QIAGEN Inc.). Real-time RT-PCR analysis of VEGF, hepatocyte growth factor (HGF), fibroblast growth factor-2 (FGF-2) and β-actin mRNA, was performed using 1 μg total RNA with ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA,

USA). mRNA levels were expressed as ratios to the level of β-actin. The following primers were designed to span genomic regions: 5'-GAG TTA AAC GAA CGT ACT TGC CAG A-3' (forward) / 5'-ATT GGA TCA GGA CCT TGT GAG-3' (reverse) for VEGF, 5'-ATT GGA TCA GGA CCT TGT GAG-3' (forward) / 5'-CCA TTC TCA TTT TGT GTG TTC A-3' (reverse) for HGF, 5'-TCT TCC TGC GCA TCC ATC-3' (forward) / 5'-TCT TCC TGC GCA TCC ATC-3' (reverse) for FGF-2, 5'-ACGTCCATT CTCCGTTTCAC-3'(forward) / 5'- TCCCACGGCC AACATAAG-3' (reverse) for GLUT1, 5'-TTGCAG TGCCTGAGTCTTCTT-3' (forward) / 5'-CCAGTC ACTCGCTGCTGA-3' (reverse) for GLUT4, and 5'-CTA AGG CCA ACC GTG AAA AG-3' (forward) / 5'-GCC TGG ATG GCT ACG ACA-3' (reverse) for β-actin.

Assay of angiogenic factors. Secreted VEGF was measured by the enzyme-linked immunosorbent assay (ELISA; R&D Systems, Mineapolis, MN, USA), which was conducted according to manufacturer's instruction.

Measurement of reactive oxygen species. ADRCs cultured under hyperglycemic condition were incubated with or without 100 μM phloretin to inhibit glucose uptake, and then incubated with 2',7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) at 1 μM (Wako) at 37°C for 30 min. The fluorescence was detected by flow cytometry.

Measurement of glucose uptake. Before starting an assay, ADRCs were starved overnight in serum free medium. Cells were then incubated in DMEM containing 25 mM 2-Deoxy-D-glucose (2DG) with or without 100 μM phloretin for 30 min. The rate of glucose uptake into ADRCs was determined by 2-h measurements using Glucose Uptake Fluorometric Assay Kit (BioVision, Inc., Milpitas, CA, USA).

Preparation of unilateral hind limb ischemia model and cell transplantation. Unilateral limb ischemia was induced in BALB/cAJcl-nu/nu mice (males, 8 ~ 10 weeks). After the mice were anesthetized by intraperitoneal infusion of xylazine hydrochloride (5 mg/kg; Bayer, Tokyo, Japan) and ketamine hydrochloride (80 mg/kg; Sankyo Pharmaceuticals, Tokyo, Japan), the proximal right external iliac artery was ligated. Immediately after producing hind limb ischemia, ADRCs (3×10^6 cells in 40 μL volume per animal) or 40 μL of saline were injected into four sites of the ischemic adductor muscle. Blood flow was evaluated 0, 2, 3 and 4 weeks after surgery using a Laser Doppler Perfusion Imager (PERIMED, Stockholm, Sweden).

Measurement of tissue capillary density. Four weeks after surgery, the ischemic adductor skeletal muscle of recipient mice was isolated, embedded in Tissue-Tek Optimal Cutting Temperature compound (Sakura Finetek Japan, Tokyo) and snap-frozen in liquid nitrogen. Cryostat sections (8 μm thick) of each tissue specimen were prepared and subjected to immunohistochemical analysis. Antibody specific for CD31 (BD Biosciences) was used and labeled by the avidin-biotin complex technique and substrates (Vector Laboratories, Burlingame, CA). The cell positive for CD31 was defined as a vascular endothelial cell. Capillary density was expressed as ratios to the skeletal muscle density. Three randomly selected fields of transverse sections per animal (n = 3 for each group) were analyzed.

Statistical analysis. All values are the mean ± standard deviation (S.D.). To assess differences among groups, data were analyzed by Student's *t*-test assuming equal variance. Other comparisons were performed using Mann-Whitney U-test with StatView 5.0 (SAS, Cary, NC, USA), where appropriate.

RESULTS

Surface markers of ADRCs cultured under normoglycemic and hyperglycemic conditions

ADRCs with surface makers CD31, CD34, CD45 and CD90 were detected by flow cytometry. Analysis of forward and side scatter characteristics indicated that ADRCs consisted of a single cell population under either normoglycemic or hyperglycemic conditions (data not shown). ADRCs, whether cultured under normoglycemic or hyperglycemic conditions, expressed lower levels of the endothelial cell marker CD31 (0.3 ± 0.1% for normoglycemic vs. 0.3 ± 0.1% for hyperglycemic; n = 5 each), endothelial progenitor cell marker CD34 (0.1 ± 0.1% for normoglycemic vs. 0.2 ± 0.1% for hyperglycemic; n = 5 each), and leukocyte marker CD45 (0.3 ± 0.1% for normoglycemic vs. 0.2 ± 0.1% for hyperglycemic; n = 5 each). Besides, they expressed a high level of the mesenchymal cell marker CD90 (95.8 ± 2.1% for normoglycemic vs. 95.4 ± 1.7% for hyperglycemic; n = 5 each).

Expression of mRNAs and proteins of angiogenic factors in ADRCs cultured under normoglycemic and hyperglycemic conditions

We determined several angiogenic factors expressed in ADRCs cultured under the normoglycemic (normal), hyperglycemic, and normoglycemic but hyperosmotic conditions. Real-time RT-PCR revealed that expression of VEGF mRNA (ratio to that of β-actin mRNA) was significantly lower in ADRCs cultured under hyperglycemic conditions (0.125 ± 0.043) than in those cultured under normoglycemic conditions (0.161 ± 0.043) or normoglycemic but hyperosmotic conditions (0.158 ± 0.034) (each n = 3) (Fig. 1A). There were no significant differences in the level of HGF and FGF-2 mRNAs among them: HGF levels were 0.00332 ± 0.00082 (normoglycemic), 0.00540 ± 0.00576 (hyperglycemic), and 0.00382 ± 0.00140 (normoglycemic but hyperosmotic); FGF levels were 0.00209 ± 0.00017 (normoglycemic), 0.00232 ± 0.00045 (hyperglycemic), and 0.00203 ± 0.00021 (normoglycemic but hyperosmotic) (each n = 3). The level of VEGF protein in cultured medium was also significantly lower when ADRCs were cultured under hyperglycemic conditions (20.434 ± 3.767 ng/mL) than when cultured under normoglycemic conditions (30.121 ± 3.923 ng/mL) or hyperosmotic but normoglycemic conditions (29.842 ± 4.729 ng/mL) (Fig. 1B).

Effects of glucose transporter inhibitors on the expression of VEGF and ROS under hyperglycemic conditions

Fig. 2A shows the mRNA levels of GLUT subtypes expressed in ADRCs under normoglycemic and hyperglycemic conditions. ADRCs expressed mRNA of GLUT1, 3 and 4, but not GLUT2. The mRNA level of any GLUT subtypes was not affected by hyperglycemic conditions. Next, the rate of glucose uptake by ADRCs was examined in the absence and presence of the glucose transporter inhibitor phloretin under normoglycemic and hyperglycemic conditions. Phloretin has been reported to equally inhibit GLUT1, 3 and 4 (8, 12, 20). Phloretin significantly decreased the rate of glucose uptake by ADRCs even under hyperglycemic conditions (Fig. 2B), while in the absence of the inhibitor the rates of glucose uptake were nearly equal under normoglycemic and hyperglycemic conditions (data not shown). As shown in Fig. 2C, the level of ROS was significantly increased under hyperglycemic conditions, with phloretin significantly decreasing ROS: ROS level determined as the fraction (%) of DCFH-DA positive cells averaged 8.1 ± 1.1 (normoglycemic), 10.7 ± 0.3 (hyperglycemic), and 6.7 ± 0.3 (hyperglycemic plus phloretin) (each n = 3). Under hyperglycemic conditions the mRNA level of VEGF was significantly decreased, and phloretin treatment significantly increased the level of VEGF mRNA (Fig. 2D): the level of VEGF mRNA as the ratio to that of β-actin mRNA averaged 0.113 ± 0.015 (normoglycemic), 0.052 ± 0.011 (hyperglycemic), and 0.079 ± 0.005 (hyperglycemic plus phloretin) (each n = 3), indicating the involvement of increased ROS in impaired production of VEGF.

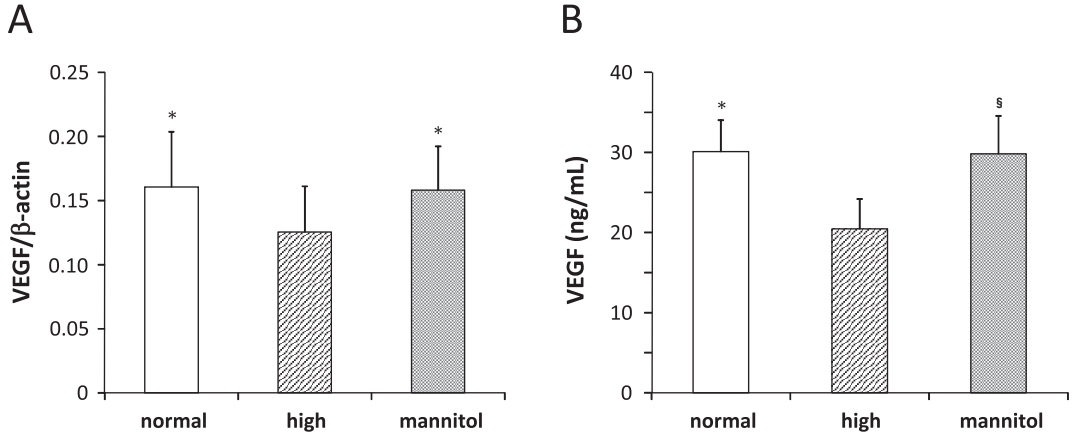

Fig. 1 Production of VEGF in ADRCs cultured in a physiological solution with 5.5 mM glucose (normal), high glucose solution with 25 mM glucose (high), or normal glucose solution containing 5.5 mM glucose and 19.5 mM mannitol (mannitol). (**A**) Real-time RT-PCR analysis of VEGF mRNA expression level, which is expressed as the ratio to *β*-actin mRNA for copy numbers of each mRNA. (**B**) Secretion of VEGF from ADRCs. Concentration of VEGF in the culture medium was measured by ELISA assay. * $P < 0.05$ vs. high glucose, § $P = 0.054$ vs. high glucose.

Effects of ADRCs implantation

To test whether ADRCs cultured under hyperglycemic conditions have angiogenic effects *in vivo*, ADRCs cultured under the normoglycemic or hyperglycemic conditions were transplanted into the ischemic hind limb of recipient mice, and angiogenesis was evaluated by blood flow and capillary density measurements. Laser Doppler perfusion imaging analysis showed that blood perfusion in the ischemic limbs was significantly improved by ADRCs cultured under normoglycemic conditions but not by ADRCs cultured under hyperglycemic conditions, as compared with the control blood perfusion without injection of ADRCs (Fig. 3). As shown in Fig. 4, the capillary density in the ischemic limb muscle where ADRCs cultured under hyperglycemic conditions were injected was significantly lower than that in the ischemic limb muscle where ADRCs cultured under normoglycemic conditions were injected, and comparable with that in control ischemic limb muscle without injection of ADRCs. Capillary density relative to the muscle bundle density at 3 different transverse sections obtained from 3 individual rats in each group averaged 0.247 ± 0.095 (without ADRCs), 0.654 ± 0.214 (normoglycemic ADRCs), and 0.200 ± 0.063 (hyperglycemic ADRCs).

DISCUSSION

The expression of VEGF by ADRCs was suppressed both at the transcription and translation levels when these cells were cultured in the presence of high concentrations of glucose. This effect was not due to hyperosmolarity, because the hyperosmotic solution containing mannitol did not affect VEGF expression. ADRCs expressing GLUT1, 3 and 4 uptake excess

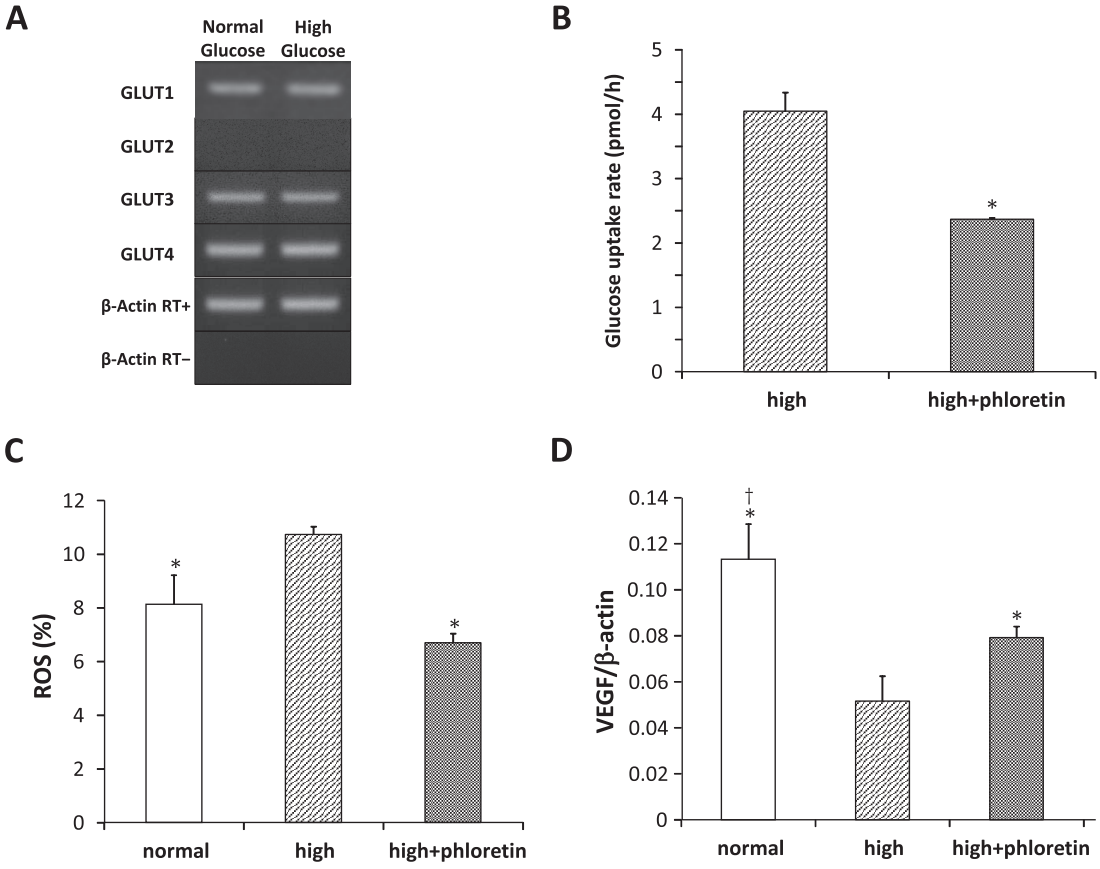

Fig. 2 Effects of hyperglycemia and glucose transporter inhibition on glucose uptake, oxidative stress, and VEGF production in ADRCs. (**A**) mRNA levels of GLUT1-4, as well as β-actin, expressed in ADRCs cultured under normoglycemic (*left*) and hyperglycemic (*right*) conditions. (**B**) The rate of glucose uptake into ADRCs in the absence and presence of phloretin, which was determined as the level of 2DG in ADRCs. (**C**) Flow cytometric analysis of ROS production detected by DCFH-DA in ADRCs with normoglycemia (normal), hyperglycemia (high), or hyperglycemia and phloretin (high + phloretin). (**D**) Real-time RT-PCR analysis of VEGF mRNA expression level, which is expressed as the ratio to β-actin mRNA for copy numbers of each mRNA. * $P < 0.05$ vs. high glucose, † $P < 0.05$ vs. high glucose and phloretin.

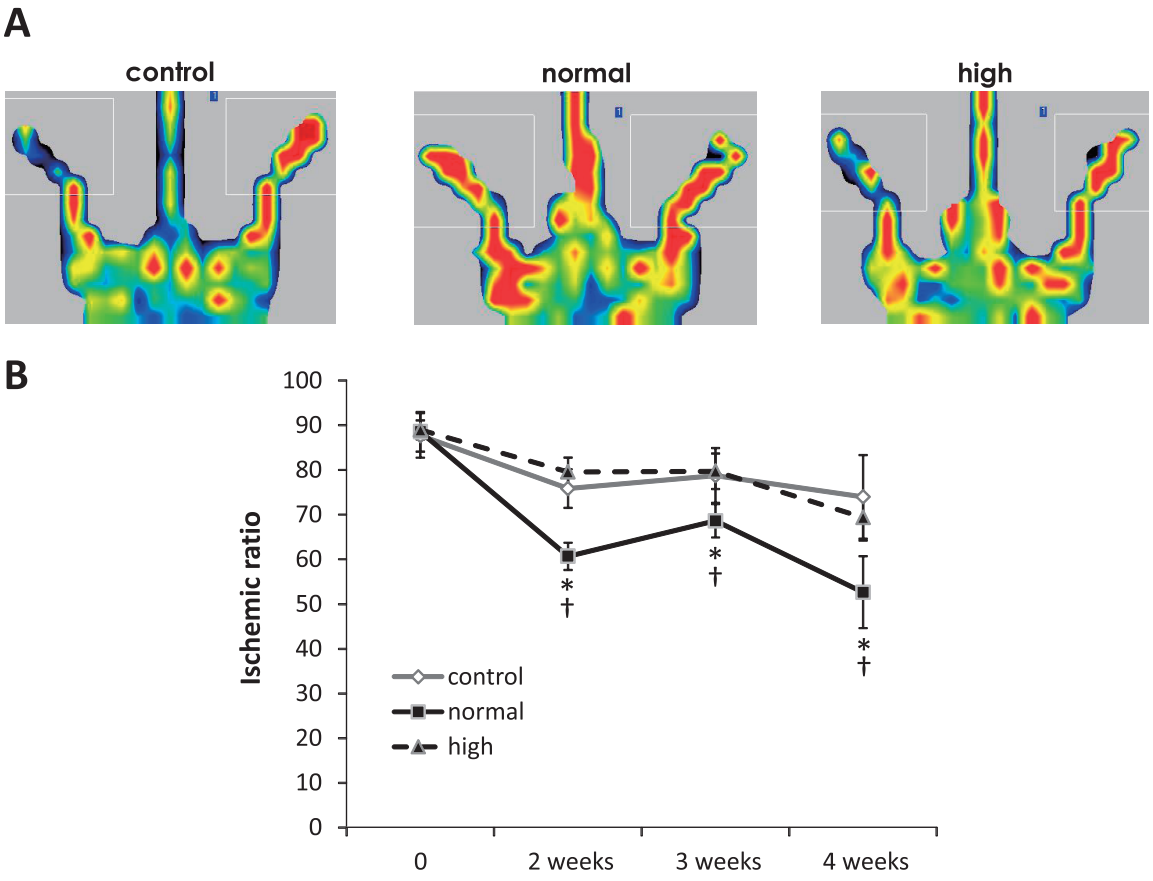

Fig. 3 Recovery of blood perfusion in ischemic hind limbs by ADRC transplantation. (**A**) Representative images of ischemic lower limbs of mice in control (control) and with transplantation of ADRCs cultured under normoglycemic (normal) or hyperglycemic (high) conditions. The images were obtained 4 weeks after ligation of the right external iliac artery (injection of ADRCs). (**B**) Averaged time-dependent recovery of blood perfusion in ischemic hind limbs. Each bar represents the mean ± S.D. control, control group; normal, injected group with ADRCs cultured under normoglycemic conditions; high, injected group with ADRCs cultured under hyperglycemic conditions. Ischemic regions were significantly reduced by injection of ADRCs cultured under normoglycemic conditions but not by injection of ADRCs cultured under hyperglycemic conditions. * $P < 0.05$ vs. control group, † $P < 0.05$ vs. high.

glucose under hyperglycemic conditions. Exposure to a high concentration of glucose increased ROS and impaired production of VEGF. The glucose transporter blocker phloretin reduced ROS and restored the expression of VEGF mRNA. As compared with normal conditions, hyperglycemic conditions impaired the angiogenic action of ADRCs transplanted into mouse ischemic limbs, decreasing both blood perfusion and the capillary density of transplanted muscle. These results indicate that a high intracellular concentration of glucose in ADRCs impairs expression of VEGF via an increase of ROS, leading to impaired proangiogenic action.

ADRCs are generally isolated from a heterogeneous mixture of stromal vascular fractions of adipose tissue by their capacity to adhere to plastic culture dishes, and they express several surface markers such as CD13, CD49, CD44, CD90, and CD105 (18). We previously found that although freshly isolated ADRCs included CD31, CD34, and CD45 positive cells, CD90 positive cells became predominant during culture, which indicated proliferation of only mesenchymal cells during culture (5). Under hyperglycemic conditions the predominance of CD90 positive cells was not affected, indicating that hyperglycemia does not influence the subpopulation of ADRCs.

There are many studies showing that the efficacy of cell transplantation therapy is mainly mediated by secretion of several angiogenic cytokines from transplanted cells (3, 14). Nakagami and coworkers (16) reported that cultured ADRCs secreted VEGF and HGF, which appear to mediate the efficacy of angiogenic cell therapy. These cytokines are well known

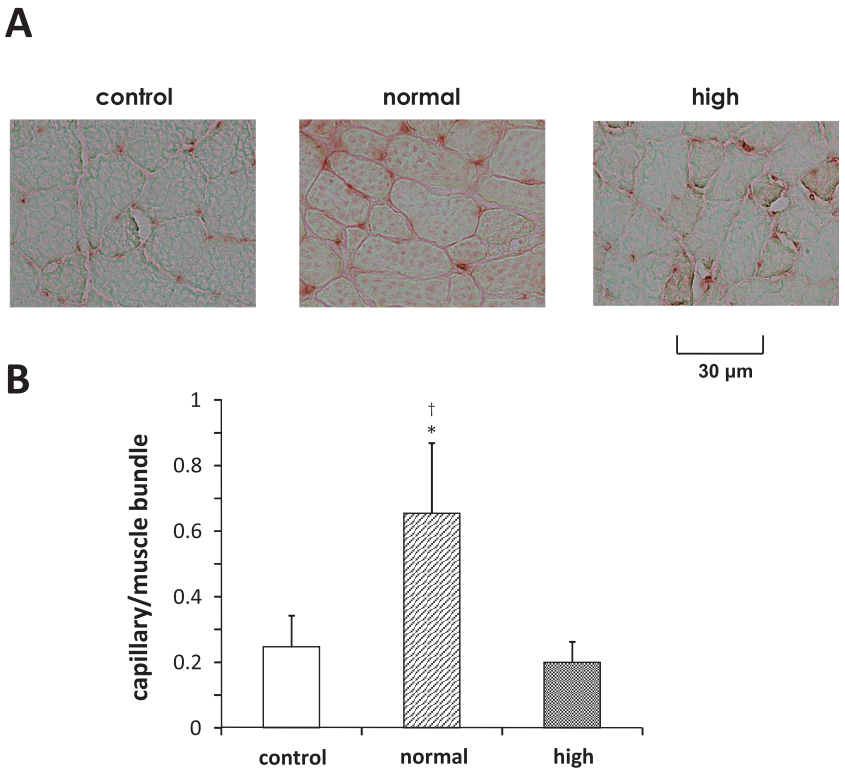

Fig. 4 Angiogenic effects of ADRC transplantation into ischemic hind limbs of mice. The data were obtained 4 weeks after ligation of the right external iliac artery (injection of ADRCs). (**A**) Representative optical micrographs of muscular capillary in the ischemic limbs transplanted with ADRCs cultured under normoglycemic (normal) and hyperglycemic (high) conditions, as well as that without ADRC transplantation (control). (**B**) Averaged capillary density as the ratio to the skeletal muscle density in ischemic limbs without ADRC injection (control), with normoglycemic ADRC injection (normal), and with hyperglycemic ADRC injection (high). The capillary density in the ischemic limb muscle injected with hyperglycemic ADRCs was significantly lower than that in the ischemic limb injected with normoglycemic ADRCs. * $P < 0.05$ vs. high glucose, † $P < 0.05$ vs. control.

to promote cell proliferation, migration, and mobilization (21). We previously found that cultured ADRCs expressed VEGF, HGF, and FGF-2 (5). It has been reported that hyperglycemia impairs the angiogenic properties of ADRCs; Kim *et al.* (9, 11) reported that rat and human adipose derived stem cells cultured in medium containing a high concentration of glucose showed reduced proliferation and an impaired proangiogenic action *in vitro*, which could be partly due to ROS generation by chronic exposure to a high concentration of glucose. ROS and reactive nitrogen species are biologically active O_2 derivatives considered as toxic molecules. Increased ROS production is recognized as a major cause of the clinical complication associated with diabetes. The mechanisms of high glucose-induced cellular dysfunction may be as follows: 1) hyperglycemia impairs the glutathione cycle to increase ROS by depleting NADPH (17); 2) hyperglycemia produces advanced glycation end products (AGEs) to thereby generate ROS via activation of AGE receptors (22); 3) hyperglycemia increases ROS via 4 individual pathways of the mitochondrial electron transport chain, NO synthase, NADPH, and xanthine oxidase activation to modulate gene expression (6); and 4) hyperglycemia reduces endothelial NO synthase phosphorylation and NO production to impair endothelial progenitor cells (EPCs) and EPC-related vascular repair (7). In the present study, the expression of VEGF mRNA and protein in ADRCs was reduced under hyperglycemic conditions leading to impairment of their angiogenic action.

The most prominent finding in the present study was that glucose transporter inhibition decreased ROS production by ADRCs under hyperglycemic conditions, restoring expression of VEGF. A high intracellular glucose concentration led to elevation of ROS and reduction of VEGF production but these were normal when cells were pretreated with phloretin. These findings indicate that hyperglycemia induces ROS via elevation of intracellular glucose concentration. It was reported that intracellular glu-

cose increased ROS through activation of glucose transporters, inducing inflammation and cellular apoptosis of vascular cells (9). However, it has never been proven that activated glucose transporters uptake excess glucose and thereby impair the angiogenic action of ADRCs. This is the first report to show the involvement of glucose transporters in hyperglycemia-induced impairment of ADRCs angiogenic action. The precise mechanisms for the involvement of subtypes of glucose transporters in ADRC-related angiogenesis need to be elucidated in future studies.

The clinical implications of our results are obvious. ADRCs cultured under hyperglycemic conditions did not increase blood perfusion nor the capillary density in transplanted muscles *in vivo*. This indicates that hyperglycemia reduces the angiogenic efficacy of ADRCs transplanted into the patients with diabetes. Lee *et al.* (11) reported that saphenous vein graft in diabetic patients yield lower colony forming unit values than that in normal individuals, which was consistent with the suppression of cell proliferation observed in a murine diabetic model (9). In the present study, VEGF production by ADRCs was impaired by a high concentration of glucose, which may explain the impaired neovascularization observed in diabetic patients. Nevertheless, this could be normalized by treatment with a blocker of glucose transporters; inhibition of glucose transporters would improve the angiogenic action of ADRCs in diabetes patients.

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