Hypoxia-enhanced wound-healing function of adipose-derived stem cells: Increase in stem cell proliferation and up-regulation of VEGF and bFGF

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ABSTRACT

Adipose-derived stem cells (ADSCs) have been shown to induce wound-healing effects. Because inflammation near the wound area induces oxygen deficiency, it is interesting to elucidate the effect of hypoxia on the function of ADSCs. In this work, we asked: (1) does hypoxia alter the wound-healing function of ADSCs? and (2) what are the major factors responsible for the alteration in the wound-healing function? Effect of hypoxia on the proliferation of ADSCs was first examined that hypoxia (2% O_2) enhanced the proliferation of ADSCs in either the presence of serum or in the absence of serum. The conditioned medium of ADSCs harvested under hypoxia (hypoCM) significantly promoted collagen synthesis and the migration of human dermal fibroblasts, compared with that in normoxia (norCM). In the animal studies, hypoCM significantly reduced the wound area compared with norCM. Furthermore, mRNA and protein measurements showed that hypoxia up-regulated growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Inhibition of VEGF and bFGF using neutralizing antibodies reversed the migration of the wounded human dermal fibroblasts and the healing of wounds in animal experiment. Collectively, these results suggest that hypoxia increases the proliferation of ADSCs and enhances the wound-healing function of ADSCs, at least partly, by up-regulating the secretion of VEGF and bFGF.

Tissue regeneration using the body's own stem cells and growth factors is a good strategy for damaged tissue (e.g., during and after inflammation). Because of few drawbacks such as ethnic consideration, adult stem cells are becoming a major candidate in stem cell medicine.[1–3] Indeed, applications of adipose-derived stem cells (ADSCs) for compensation of skin defects have shown some satisfactory results.[2,4,5] Not only can ADSCs reconstruct a tissue, but they also have the ability to control or cure other cells and can reconstruct an integrated function. For example, we have previously demonstrated that ADSCs accelerated wound healing by secreting growth factors.[6] In that study, a conditioned medium of ADSCs (ADSC-CM) activated collagen synthesis and migration of human dermal fibroblasts (HDFs), thus accelerating wound healing. In addition, ADSCs exhibited an antioxidant effect and protected HDFs from oxidative stress,[7] suggesting that ADSCs play a unique role in tissue protection in hypoxic/oxidative stress. However, little is known regarding the function of ADSCs under hypoxia. To better understand the mechanism of ADSCs-induced tissue protection from wound healing, it is important to clarify this issue because inflammation and oxidative stress near the wound area induce an oxygen deficit.[8,9]

Oxygen is a potent signaling molecule that has received increasing recognition for its ability to affect the fundamental characteristics of various cells. A state of oxygen deficiency, hypoxia, may cause an impairment of function. When the cell is unable to extract adequate oxygen, the partial pressure of oxygen within the cell declines, which leads to a reduction in mitochondrial respiration and oxidative metabolism. However, cellular responses to hypoxic stress considerably depend on the cell types, maturity, and environmental conditions. For example, in vivo mesenchymal stem cells (MSCs) reside in a hypoxic region in our body and when they are cultured under hypoxic conditions in vitro, their proliferative and self-renewal capacities are significantly improved.[10–13] Clearly, a reduction in the tension of atmospheric oxygen may significantly alter the metabolism of stem cells. Several lines of evidence have suggested that hypoxia amplifies the response of ADSCs to pharmacological agents via a paracrine mechanism.[14–17] An increase in the secretion of potent growth factors from ADSCs may partially account for the enhanced activity.[14–16] However, there appears to be conflicting results from studies concerning the function of ADSCs. For instance, a report shows that exposure of ADSCs to low oxygen for a period of 2 weeks reduced their proliferation although it

induced a two-fold increase in the rate of protein synthesis and a three-fold increase in total collagen synthesis.[18]

This work was performed to clarify the functional changes in ADSCs during hypoxia. We hypothesized that hypoxia will affect cell proliferation and protein secretion of ADSCs, and thus affect their response to pharmacological agents in the skin. We cultured ADSCs in hypoxia and then evaluated their proliferation and survival and compared the data with those of normoxia. In addition, ADSC-mediated wound-healing function was studied in the conditioned medium collected under a hypoxic or a normoxic condition. Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) was performed to measure mRNA expression of hypoxic ADSCs, and an enzyme-linked immunosorbent assay (ELISA) was used to quantify the secreted protein levels in the conditioned medium. Finally, we studied the effect of neutralizing antibodies against vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in the animal study. Our data suggest that hypoxia increases the proliferation of ADSCs and enhances the wound-healing function of ADSCs by up-regulating VEGF and bFGF.

MATERIALS AND METHODS

Isolation and culture of ADSCs and HDFs

Human subcutaneous adipose tissue samples were acquired from elective liposuction of healthy females with informed consent as approved by the institutional review boards. The obtained samples were digested with 0.075% collagenase type II (Sigma-Aldrich, St. Louis, MO) under gentle agitation for 45 minutes at 37 °C, and centrifuged at $300\times g$ for 10 minutes to obtain the stromal cell fraction. The pellet was filtered with a 70 μm nylon mesh filter, and resuspended in phosphate-buffered saline. The cell suspension was layered onto histopaque-1077 (Sigma-Aldrich), and centrifuged at $840\times g$ for 10 minutes. The supernatant was discarded, and the cell band buoyant over histopaque was collected. The retrieved cell fraction was cultured overnight at 37 °C/5% CO_2 in control medium (Dulbecco's modified Eagle's media [DMEM], 10% fetal bovine serum [FBS], 100 U/mL of penicillin, and 100 μg/mL of streptomycin). The resulting cell population was maintained over 3–5 days until confluence, which were represented as passage 1. ADSCs were cultured and expanded in control medium, and used for the experiments at passages 4 through 5. Characterization of ADSC has been described previously[6,7,19] Punch biopsies were performed for sampling HDFs, which were then isolated and cultured as described previously.[20]

Survival and proliferation of ADSCs

ADSCs (2×10^3 cells/well) were seeded in 48-well plates with a serum-free medium and a serum-containing medium (10% FBS) of DMEM and DMEM/F12. ADSCs were incubated under normoxia (20% O_2, 5% CO_2) and hypoxia (2% O_2, 20% CO_2, and balanced N_2) for 24, 48, and 72 hours, respectively. The morphology of ADSCs was monitored every day and the cell number was measured using the CCK-8 assay kit (Dojindo, Gaithersburg, MD). ADSCs were mixed with 20 μL of the CCK-8 solution. The absorbance was measured at 450 nm using a microplate reader (Tecan, Grödig, Austria). The OD values of each well were measured to represent the survival/proliferation of ADSCs. All the experiments were performed in triplicate.

Preparation of ADSC-CM

ADSCs were cultured and expanded with FBS (10%) for a total of four passages under normoxic conditions. Then, ADSCs (4×10^5 cells) were seeded in 100 mm culture dishes. When the ADSCs reached confluence, the medium was changed to a DMEM/F12 serum-free medium (Invitrogen–Gibco-BRL, Grand Island, NY). After changing the medium, ADSCs were exposed to hypoxia (2% O_2, 5% CO_2, and balanced N_2) for 72 hours. Control cultures were incubated in 95% room air and 5% CO_2. Then, conditioned media of ADSCs in nomoxia (norCM) and in hypoxia (hypoCM) were collected, centrifuged at $300\times g$ for 5 minutes, and finally filtered using a 0.22 μm syringe filter.

Measurement of type I collagen secreted by HDFs

HDFs (2×10^4 cells/well) were seeded in 24-well plates and cultured overnight in DMEM with 0.1% FBS. Then, culture medium was changed to either norCM or hypoCM, followed by incubation for 72 hours. Aliquots of culture medium were separated using an 8% sodium dodecyl sulfate-polyacryamide gel, and then blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were incubated with type I collagen primary antibody (1 : 1,000, Santa Cruz Biotechnology, Santa Cruz, CA), washed, and incubated with a secondary antibody conjugated to horseradish peroxidase (1 : 10,000, Zymed, San Francisco, CA). Immunoreactive bands were detected using a chemiluminescent substrate system (Immunobilon Western reagent; Millipore), and quantified using a densitometer and GeneTools software (Syngene, Cambridge, UK).

Proliferation of HDFs

HDFs (3×10^3 cells/well) were seeded in 48-well plates with a serum-free medium. After overnight attachment, the cells were incubated in the medium containing 100% norCM or hypoCM for 24 and 48 hours. The cell number was measured using the CCK-8 assay kit (Dojindo). Absorbance was measured at 450 nm using a microplate reader (Tecan). The OD values of each well were measured to represent the proliferation of HDFs. All the experiments were performed in triplicate.

Assay of HDF migration

For the measurement of cell migration, confluent HDFs were kept in a serum-free medium for 24 hours and wounded with a plastic micropipette tip with a large orifice. After washing, the medium was replaced either with norCM or with hypoCM. Photographs of the wounded

area were taken every 24 hours by phase-contrast microscopy. For evaluation of wound closure, four randomly selected points along each wound were marked, and the horizontal distance of migrating cells from the initial wound was measured.

Animal experiments

Four female hairless mice (Orient Bio, Sungnam, Korea) were used in animal experiments. Experimental protocols involving the mice used in this study were reviewed by the Animal Care and Use Committee in the College of Pharmacy, Seoul National University, according to the National Institutes of Health (NIH) guidelines (NIH publication number 85-23, revised 1985) for the Principles of Laboratory Animal Care.

On the day of operation, the mice were anesthetized by a subcutaneous injection of a mixture of ketamine (20 mg/kg) and acepromazine (10 mg/kg). Two circular full-thickness wounds of 8 mm diameter were created on the backs of the mice. NorCM (left side of the back) and hypoCM (right side of the back) in a collagen gel mixture were placed in the wounds, which were dressed with transparent Tegaderm® (3M Health Care, St. Paul, MN). Animal behavior and bandage integrity were monitored during the experiment. Wounds were evaluated 4 and 7 days after surgery. Digital pictures were taken to visualize the wound. Wound healing was quantitatively measured and calculated by the remaining wound area.

Analysis of ADSCs by Q-RT-PCR

ADSCs were exposed to hypoxia in 2% O_2, 5% CO_2, and balanced N_2 for 6 hours. Total cellular RNA was extracted using an Easy-spin™ kit (iNtRON Biotechology, Seoul, Korea), followed by a reverse transcription using a cDNA synthesis kit (Promega, Madison, WI). cDNA was synthesized from 1 μg total RNA, using 200 U of reverse transcriptase (M-MLV RT) and 100 pM oligodT. Table 1 shows the oligonucleotides that were used as primers and the internal control β-actin. RT-PCRs were performed in a final volume of 20 μL reaction mix that contained 10 μL of the SYBR Premix Ex Taq™ II (Takara Bio Inc., Otsu, Japan) and 1 μL of each primer set using the LightCycler 480 II (Roche Diagnostics Corp., Indianapolis, IN), with the following cycling program: 30 seconds at 94 °C, 30 seconds at 56 °C, and 60 seconds at 72 °C. The β-actin mRNA level was used for sample standardization. All the experiments were performed in duplicate and repeated two times.

ELISA assay of conditioned medium

The concentrations of several cytokines and active proteins involving wound healing in both norCM and hypoCM were measured using sandwich ELISA kits according to the manufacturer's instructions: VEGF, bFGF, transforming growth factor-β1 (TGF-β1), insulin-like growth factor (IGF), keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF)-A, type I collagen, and fibronectin were obtained from R&D Systems (Minneapolis, MN). All the experiments were performed in duplicate.

Table 1. Primer sequences used in the quantitative reverse transcription-polymerase chain reaction

Gene		Primer sequence	Fragment (bp)
bFGF	F	5′-TGCTGGTGATGGGAGTTGTA-3′	482
	R	5′-CCTCCAAGTAGCAGCCAAAG-3′	
VEGF	F	5′-TACCTCCACCATGCCAAGT-3′	343
	R	5′-TGCATTCACATTTGTTGTGC-3′	
IGF	F	5′-TGTCCTCCTCGCATCTCTTC-3′	357
	R	5′-CACTCCCTCTACTTGCGTTC-3′	
KGF	F	5′-TGCCAACTTTGCTCTACAG-3′	318
	R	5′-CACTTTCCACCCCTTTGA-3′	
PDGF-A	F	5′-TAGGGAGTGAGGATTCTTTGG-3′	373
	R	5′-CACTCCAAATGCTCCTCTAAC-3′	
TGF-β1	F	5′-GGAAACCCACAACGAAATC-3′	381
	R	5′-GTAGTGAACCCGTTGATGTCC-3′	
Fibronectin	F	5′-TGAAGAGGGGCACATGCTGA-3′	274
	R	5′-GTGGGAGTTGGGCTGACTCG-3′	
Collagen I	F	5′-CCCTCAAGGTTTCCAAGGAC-3′	280
	R	5′-ACCAGGTTCACCCTTCACAC-3′	
β-Actin	F	5′-ACCCTGAAGTACCCCATCG-3′	274
	R	5′-CACCGGAGTCCATCACG-3′	

bFGF, basic fibroblast growth factor; IGF, insulin-like growth factor; KGF, keratinocyte growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

Functional inhibition of VEGF and bFGF

HDFs (4×10^5 cells/well) were seeded in a six-well plate with serum-free DMEM media overnight. At the time of 100% confluence, HDFs were wounded using a plastic micropipette tip with a large orifice. After several washings, the media were changed to ADSC hypoCM without or with growth factor antibody. Neutralizing antibodies of bFGF and VEGF were applied at a 1 μg/mL concentration in this experiment (R&D Systems). All the experiments were performed in duplicate.

For in vivo experiments, we produced full-thickness wounds by an 8 mm punch biopsy on both sides of mice dorsal midline (n=4). After the injury, wounds were treated with hypoCM (100 μL) on the left side of the back and hypoCM pretreated with neutralizing antibody (VEGF [1 μg/mL] and bFGF [1 μg/mL]) on the right side of back. Wound areas were measured with digital calipers 4 and 7 days after surgery.

Statistical analysis

Data are representative of three or more independent experiments. $p < 0.05$ or $p < 0.01$ are considered to be significant.

RESULTS

Survival and proliferation of ADSCs

As shown in Figure 1, ADSCs cultured in hypoxia exhibited better survival/proliferation than ADSCs in normoxia in the complete DMEM medium. This phenomenon could also be seen when they were cultured in DMEM/F12 medium. In addition, the survival/proliferation of ADSCs was studied in a serum-free condition, and it was found that the rate of survival/proliferation of ADSC was slightly increased by 72-hour hypoxia (2%) in serum-free DMEM/F12 medium, but was not significantly different under other conditions.

Hypoxia-enhanced wound-healing function of ADSCs

Because serum contains diverse growth factors that accelerate wound healing, serum-free conditioned medium was harvested to verify the wound-healing function of ADSCs. It has been previously demonstrated that ADSC accelerated wound healing by increasing the collagen synthesis and promoting the migration of HDF 6. Therefore, collagen synthesis and migration of HDFs were compared in vitro using hypoCM and norCM. As shown in Figure 2, hypoCM had a more potent effect on type I collagen secretion by HDFs than did norCM. However, there was no significant difference in the proliferation of HDFs between the norCM group and that of hypoCM (Figure 2C). In addition, hypoCM enhanced migration of HDFs by approximately 1.5-fold over norCM ($p < 0.01$) after a 48-hour incubation (Figure 3).

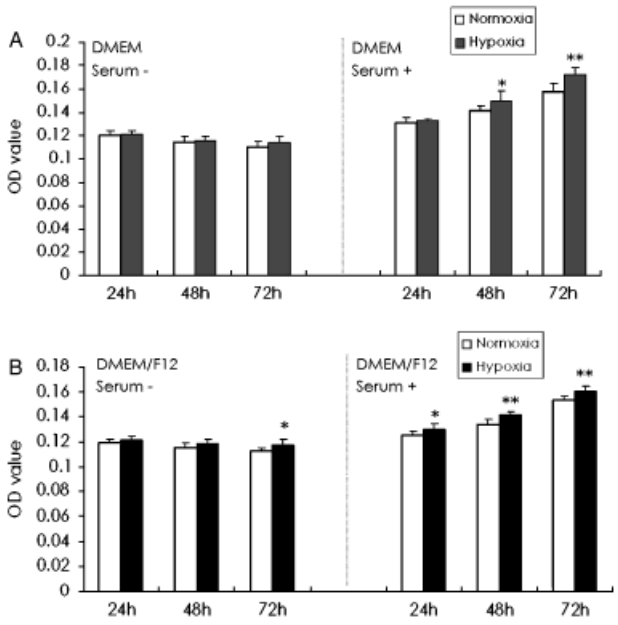

Figure 1. Proliferation of adipose-derived stem cells (ADSCs) in Dulbecco's modified Eagle's media (DMEM) (A) and DMEM/F12 medium (B). Proliferation of ADSCs was examined in the presence or absence of serum in hypoxia (closed bars) or normoxia (open bars). Note that survival and proliferation of ADSCs was increased by hypoxia. *$p < 0.05$, **$p < 0.01$ compared with each normoxia group.

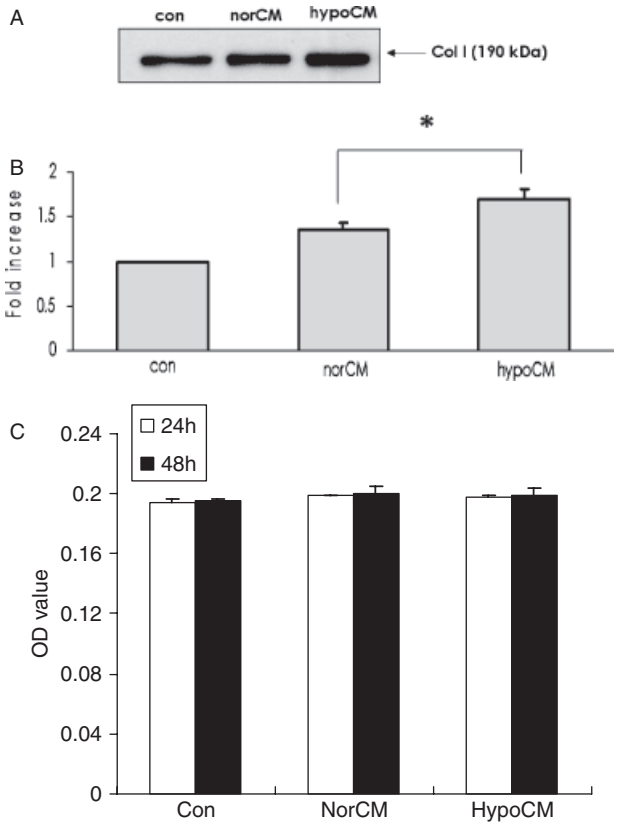

Figure 2. Western blot of collagen type I secreted from human dermal fibroblasts (HDFs) with the addition of DMEM/F12 serum-free medium (con), conditioned medium of adipose-derived stem cells in normoxia (norCM) and hypoxia (hypoCM) (A). The signal density was measured using a densitometer (B). The proliferation of HDFs was measured after treatment of norCM and hypoCM, but was not different (C). Note that the band density of collagen type I was increased by hypoCM. *$p < 0.05$. DMEM, Dulbecco's modified Eagle's media.

Furthermore, we compared the wound-healing function of hypoCM and norCM in the animal studies. Similar to in vitro studies, the treatment of hypoCM had a superior effect to that of norCM. Four days after surgery, hypoCM treatment significantly reduced the size of the wound area as well as their depth compared with norCM (Figure 4A). The size of the wound area was reduced by 27% ($p < 0.01$, $n=4$) (Figure 4B). In addition, wound closure was much faster in the hypoCM than in the norCM.

Hypoxia regulation of specific mRNA levels in ADSCs

Q-RT-PCR was performed to assess the hypoxia-induced expression of growth factors and proteins involved in wound-healing in ADSCs. As shown in Figure 5, hypoxia differentially up-regulated the mRNA expression of

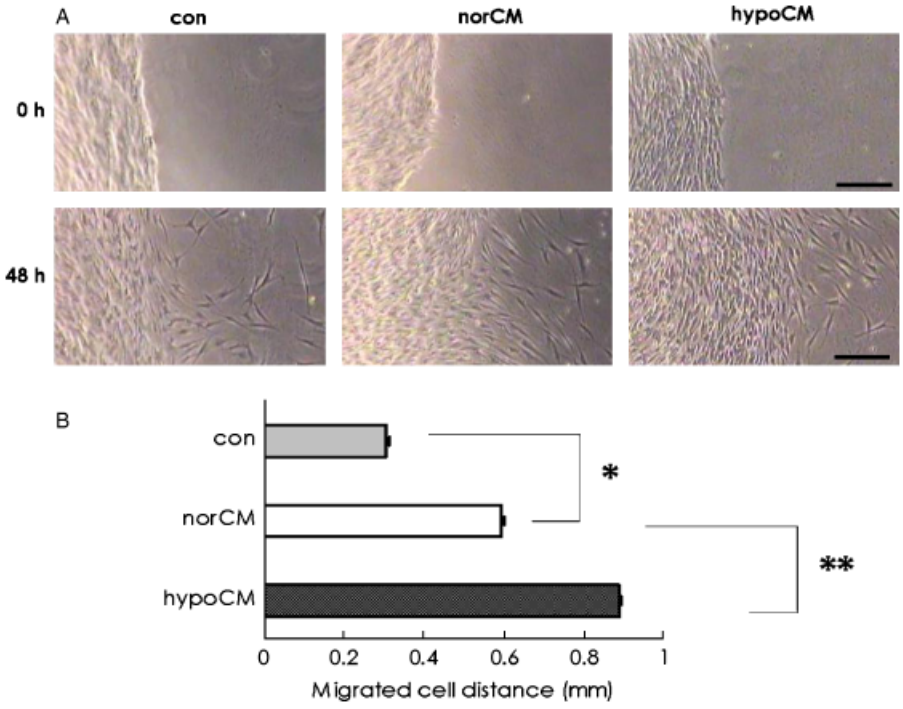

Figure 3. Human dermal fibroblast (HDF) migration assay. Monolayers of confluent HDFs were wounded using plastic micropipette tips, and then treated with DMEM/F12 serum-free medium (con), nor-CM, and hypo-CM. Photographs were taken 48 hours after wounding (A) and the migrated length was measured (B). Note that migration was increased by hypoCM. Scale bar=100 μm. *$p < 0.05$, **$p < 0.01$. DMEM, Dulbecco's modified Eagle's media.

VEGF, bFGF, and IGF-1. The change in other mRNA expressions such as collagens, fibronectin, TGF-β1, KGF, and PDGF-A was not statistically different.

Increased secretion of active proteins

Secreted proteins in the hypoCM or norCM were measured and compared using the method of ELISA. The results of Table 2 showed a significant up-regulation of VEGF (2.8-fold) and bFGF (2.3-fold). The mRNA level of IGF was increased in early time of hypoxia; however, protein levels were not detected in our ELISA assay system. This inconsistency may be due to the low protein level of IGF under our culture condition. Although IGF level was highly expressed in serum-containing medium (data not shown); the IGF level was not measured in the serum-free conditions in our assay system. Concentrations of other proteins were not changed by hypoxia.

Inhibition of ADSC-CM by neutralizing antibodies of VEGF and bFGF

Because hypoxia significantly increased the secretion of VEGF and bFGF, it was hypothesized that they may be responsible for the enhancement of wound-healing function by hypoxia. Therefore, we neutralized VEGF and bFGF by specific antibodies and then investigated the migration of HDFs (Figure 6A). The migration of HDF was slightly reduced by bFGF. However, the migration of HDFs was significantly decreased by the addition of neutralizing antibodies against VEGF. The inhibitory effect was increased by cotreatment of these two antibodies (approximately 61% reduction, Figure 6B). In addition to an in vitro experiment, the wound-healing effect of hypoCM was significantly reduced by the addition of the antibodies of both VEGF and bFGF in the animal experiment (approximately 31% reduction 4 days after surgery, Figure 7). Therefore, VEGF and bFGF may, at least partly, play a role in the enhancement of wound-healing function by hypoxia.

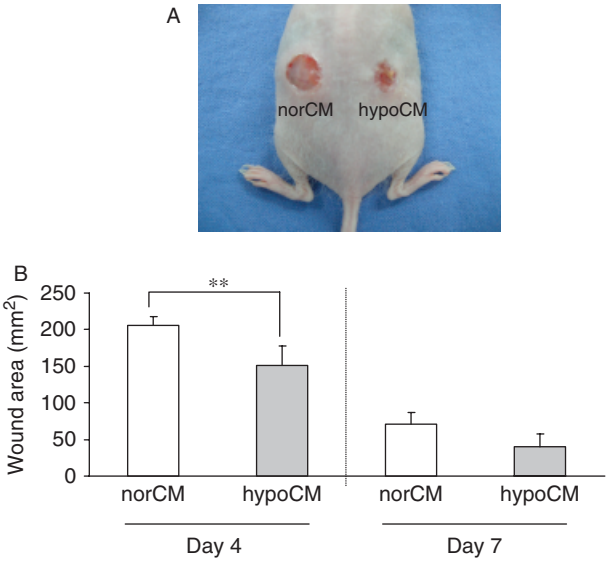

Figure 4. Animal experiment. Treatment with hypoCM significantly reduced the size of the wound area and wound depth compared with that of norCM (A). Wound at 4 days after treatment (B). Quantitative measurement of the size of the wound area. Note that the wound area was reduced approximately 27% by hypoCM. **$p < 0.01$.

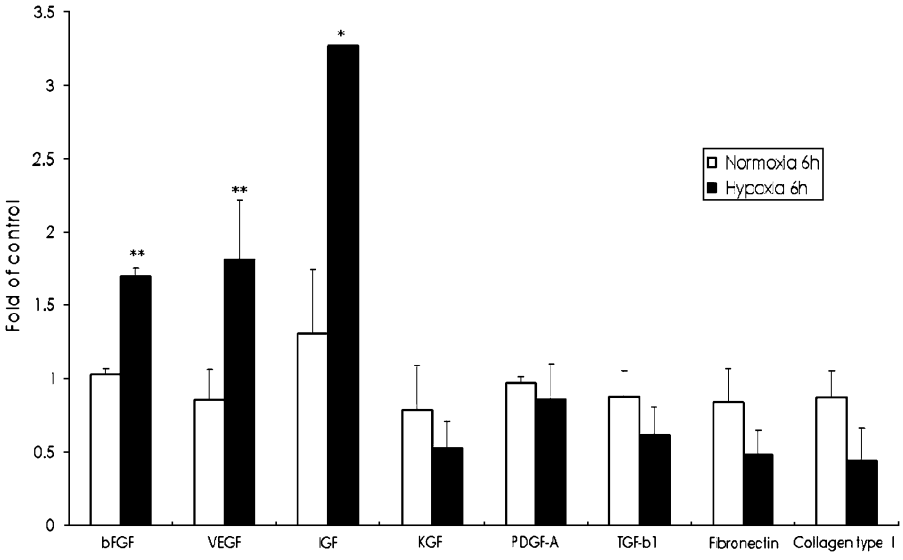

Figure 5. mRNA expression of growth factors and specific proteins involved in wound healing in normoxia and hypoxia. The value of data was calculated by comparing with the normalized actin. Note that the expression of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF) was significantly increased 6 hours after hypoxia. $*p < 0.05$, $**p < 0.01$ compared with each factor expression level in normoxia.

DISCUSSION

Our work showed that the function of ADSCs involved in wound healing was significantly enhanced by hypoxia and the proliferation of ADSCs was significantly increased during hypoxia. In the serum-free condition, specific growth factors involved in the wound-healing process (e.g., VEGF and bFGF) were up-regulated by oxygen deficiency. In addition, wound healing enhanced by hypoxia was reversed by neutralizing antibodies against VEGF or bFGF in an animal experiment and in vitro migration model. These results collectively demonstrate that hypoxia enhanced the wound-healing function of ADSCs by increasing the proliferation of ADSCs and up-regulation of growth factors, suggesting a significant role of ADSCs in the wound-healing process.

ADSCs are important stromal cells that repair the injured tissue and migrate in order to interact with other cells and with the extracellular milieu according to different stimuli. Under physiological as well as under pathologic conditions, ADSCs may be frequently found within the stromal-vascular fraction of subcutaneous adipose tissue, where they can easily access oxygen. However, there are several other circumstances, such as during wound healing, in which capillary injury generates a hypoxic environment. ADSCs' response to hypoxic condition and regeneration of wounds by ADSCs are accelerated. Our experimental findings support the building-block function that ADSCs play a significant role in the response to injury, being capable of high proliferation under hypoxic conditions. In addition to the building-block function of ADSCs, our expression studies on the growth factors and inhibition studies by neutralizing antibodies clearly showed the paracrine effect of ADSCs. During hypoxia, secretions of growth factors such as VEGF and bFGF are increased from ADSCs, which activate the fibroblasts (collagen synthesis and migration of fibroblasts are increased) and accelerate the healing of wounds.

Table 2. Protein concentration secreted

Secreted protein	Normoxia	Hypoxia	Unit
bFGF*	10.62 ± 12.75	24.75 ± 9.11	pg/mL
VEGF**	70.17 ± 16.07	200.17 ± 35.47	
IGF	Not detected	Not detected	
KGF	39.97 ± 5.29	40.31 ± 2.89	
PDGF	72.27 ± 16.71	82.29 ± 18.43	
TGF-β1	120.54 ± 3.02	143.24 ± 18.38	
Fibronectin	2,672.71 ± 201.63	2,787.18 ± 147.69	ng/mL
Type I collagen	733.25 ± 170.87	820.77 ± 70.99	

$*p < 0.05$, $**p < 0.01$.
bFGF, basic fibroblast growth factor; IGF, insulin-like growth factor; KGF, keratinocyte growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

Proliferation of ADSCs was significantly increased under the hypoxic culture condition in this examination. In addition, several reports demonstrated that stem cells reside in our body and in vitro have enhanced self-renewal capacities under hypoxic conditions. For example, hyperbaric oxygen induced endogenous neural stem cells to proliferate and differentiate in hypoxic–ischemic brain damage in neonatal rats, which contributed to repair of the injured brain.[21] Isolation of MSCs by gravity sedimentation, together with culture medium supplementation with 5% of platelet lysate in a hypoxic atmosphere (5% O_2), significantly improved MSC yield and reduced the expansion time compared with the standard accepted protocols.[22] Lennon et al.[11] found that primary rat MSCs exhibited higher proliferation rates and enhanced differentiation characteristics when grown under hypoxic conditions. Although there was a contrasting result that ADSCs showed a lower proliferation rate in 5% hypoxia,[23] it is reasonable to assume that hypoxia in the wound

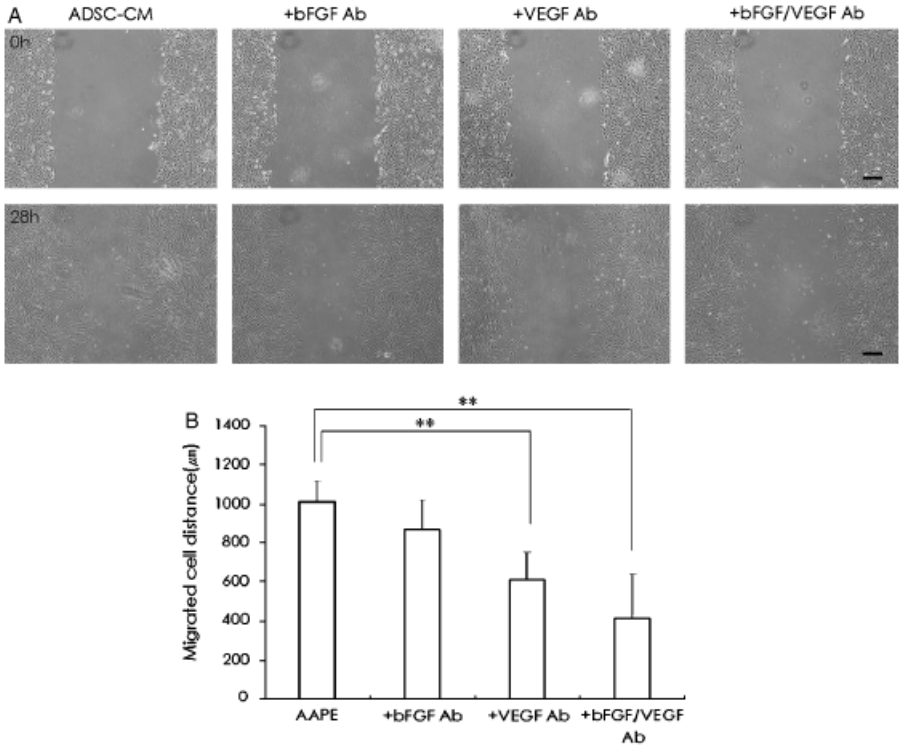

Figure 6. Functional inhibition of a conditioned medium of ADSC (ADSC-CM) by neutralizing antibodies of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in human dermal fibroblast (HDF) migration (A), and the migrated distance was measured (B). Note that the migration of HDFs was decreased by the addition of blocking antibodies of VEGF and bFGF and the inhibition rate was increased by co-treatment. Scale bar=400 μm. $^{**}p < 0.01$. ADSC, adipose-derived stem cells.

milieu enhances the proliferation of ADSCs and this may accelerate the healing of wounds in the skin.

Hypoxia enhances the function of ADSCs via a paracrine mechanism, with the secretion of certain growth factors being elevated.[14,15,17] For example, ADSCs improved perfusion in hindlimb ischemia induced by femoral artery ligation and the function of ADSCs was enhanced by hypoxic conditions. In that study, a particular growth factor that played an essential role in the cellular response to hypoxia was VEGF, which induced angiogenesis and antiapoptosis.[14] Gnecchi et al.[24,25] observed that the paracrine actions of the stem cells through the release of soluble factors were important mechanisms for tissue repair and functional improvement after injection of the Akt-transfected MSCs. In these studies, several genes coding for VEGF, bFGF, and IGF were significantly up-regulated in response to hypoxia. These growth factors promoted tissue repair in infarcted myocardium. In an in vitro study, the migration and tube formation of bone marrow-derived MSCs were induced by growth factor-enriched conditioned media of hypoxic culture conditions, suggesting paracrine-regulatory mechanisms.[26] Therefore, it is likely that up-regulation of growth factors such as VEGF and bFGF under hypoxic culture conditions may account for the increased function of ADSCs involving wound healing in this study.

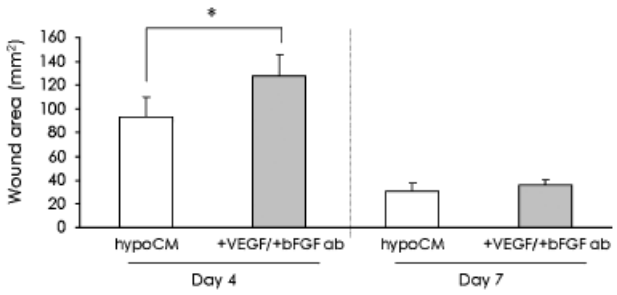

Figure 7. Inhibition of the wound-healing effect of hypoCM by neutralizing antibodies against vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in an animal experiment. Note that the wound area was significantly increased by blocking antibodies against both VEGF and bFGF. $^{*}p < 0.05$.

In summary, hypoxia increased the proliferation of ADSCs and enhanced the wound-healing function of ADSCs in in vitro and animal studies. The mechanism may be related to the hypoxia induced up-regulation of specific growth factors such as VEGF and bFGF in ADSCs.

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