Low Oxygen Tension Enhances Proliferation and Maintains Stemness of Adipose Tissue–Derived Stromal Cells

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Abstract

Recent evidence indicates that low oxygen tension or hypoxia alters the characteristics of stem cells. The actions of hypoxia are mediated through the hypoxia-inducible factor, a critical mediator of the cellular response to hypoxia. Adipose tissue–derived stromal cells (ASCs) are one of the most promising cell sources for tissue engineering applications. This study investigated the effect of hypoxia on ASCs in terms of the ability to proliferate and differentiate. ASCs were extracted from mice and maintained under hypoxic atmosphere (2% O2) for up to eight in vitro passages. The proliferation rate was examined as a growth curve, and the potency of differentiation was evaluated. To investigate the cell characteristics, we checked several stem-cell markers and growth factors. Compared with the normoxic state (20% O2), hypoxia enhances proliferation with an approximately six- to sevenfold higher ASC expansion over 6 weeks. The expression of Oct3/4 and Nanog (stem-cell marker) and the amount of secreted growth factors were increased under the hypoxic condition. These results suggest that low oxygen tension enhances proliferation and maintains stemness of ASCs. Thus, this study emphasizes the profitability of hypoxic culture for expansion of ASCs and maintenance of their undifferentiated state for further therapeutic use.

Key words: cell growth; differentiation; hypoxia; hypoxia-inducible factor; stem cell

Introduction

Recent studies have revealed that low oxygen tension or hypoxia is an important factor to alter characteristics in various types of stem cells, such as embryonic stem (ES) cells,1 induced pluripotent stem (iPS) cells,2 and some adult somatic stem cells.3-5 A low oxygen environment is physiologic not only for most mammalian embryos but also for adult somatic stem cells, while ambient air oxygen tension of 21% O2 is not a physiological condition. Such a low oxygen tension is also called "physiological normoxia."6 Hypoxia-inducible factor (HIF) plays a crucial role in hypoxic signal transduction and regulates the development or differentiation physiologically.7-9

Adipose tissue is easily available through the high abundance of cosmetic surgeries for fat removal and a rich source of adipose tissue–derived stromal cells (ASCs). ASCs are mesenchymal stem cells with self-renewal capacity and potential for differentiation and multilineage plasticity including bone,10 fat,11 and neural tissue.12 ASCs and bone marrow–derived stromal cells (BMSCs) share similar surface markers, gene profiles, and functions.13 These somatic stem cells, unlike ES or iPS cells, have no risk of forming tumors like teratoma. Compared with BMSCs, ASCs are advantageous in that extracting adipose tissue is less invasive and the tissue is more easily harvested. Moreover, the volume of ASCs obtained by one surgery is much higher. Therefore, ASCs are one of the most promising cell sources for tissue engineering applications.14 In addition, an essential function of ASCs is the production and secretion of growth factors that activate angiogenesis. These growth factors include vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).15 ASCs are superior to BMSCs in terms of production of VEGF and HGF.

Since ASCs are a type of adult somatic stem cell, they are inferior to ES or iPS cells in terms of proliferative and differentiation ability. In considering the application of ASCs in tissue engineering, the superior ability to proliferate and differentiate is a benefit. In vivo measurements in mouse adipose tissues have shown the oxygen concentrations to be in

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the vicinity of ~3% despite the high degree of vascularity in fat. The hypoxic condition may still be beneficial for ASC in vitro proliferation and differentiation characteristics; however, the role of hypoxia on ASCs has not been fully elucidated.

We hypothesized that hypoxic conditions are beneficial for ASC proliferation and maintenance of an undifferentiated phenotype “stemness” during monolayer culture. For this study, we obtained ASCs primarily from fat tissue and maintained them under hypoxic atmosphere (2% O₂) for up to eight in vitro passages. We then examined whether low oxygen tension can enhance proliferation and maintain the stemness of ASCs.

Materials and Methods

Cell harvest and culture

This study was conducted according to the guidelines of the Institutional Review Board for the Care of Animal Subjects at the National Defense Medical College, Japan. Murine ASCs were isolated as previously described. Briefly, the inguinal adipose tissue of 5-week-old C57BL/6J mice (Japan SLC) was extracted and washed extensively with Dulbecco’s modified Eagle’s medium (DMEM). The adipose tissue was digested for 2 h at 37°C with 0.1% collagenase type I (Wako Pure Chemical Industries). The cells were treated with a 40-μm nylon mesh (Becton Dickinson), and then collected by centrifugation at 3000 rpm = 1800 g for 10 min. After removing the supernatant, the cells were resuspended in DMEM containing 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin; control medium). The yield of ASCs was estimated to be more than 5 x 10⁶ cells/g of adipose tissue obtained from one mouse. The experiments were performed three times. ASCs were cultured at 37°C and 5% CO₂. For normoxic cultures, ASCs were cultured at 95% air (20% O₂)-5% CO₂. For hypoxia studies, ASCs were cultured in a multigas incubator (ASTEC) that was flushed with humidified gas mixture composed of 2% O₂:5% CO₂:93% N₂.

Growth potential

To assess growth potential, passage 1 (P1) ASCs were thawed, counted, and plated onto a 100-mm dish at 1 x 10⁵ cells/dish. Three dishes were placed under normoxic conditions and another three under hypoxic conditions. The cells were allowed to proliferate for 5 days (P1–P4) and 7 days (P5–P8) and then samples were harvested with 0.05% trypsin. We then examined whether low oxygen tension can enhance proliferation and maintain the stemness of ASCs.

Flow cytometry

We harvested adherent ASCs and resuspended them at 1 x 10⁶ cells/mL in Dulbecco’s phosphate-buffered saline (DPBS). We incubated cells with murine specific monoclonal antibodies directed against CD29, CD45, CD117 (c-kit), CD34, and Sca-1. Antibodies were coupled to either R-phycocerythrin or fluorescein isothiocyanate. We analyzed the cells using a FACSCalibur Cytometer (Becton Dickinson). We obtained all antibodies from eBioscience.

Western blotting

After growing to confluence, ASCs were scraped off and collected using 2 x 10⁶ SDS sample buffer (125 mM Tris-HCl [pH 6.8], 4% w/v SDS, and 20% glycerol). Protein concentrations were measured by a commercial kit (DC Protein Assay Kit; Bio-Rad Laboratories) using bovine serum albumin as the standard. Protein samples (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride microporous membrane (Immobilon-P; Millipore). The membrane was blocked with Block Ace® (DS Pharma Biomedical Co.) for 1 h at room temperature, and then incubated overnight at 4°C with the primary antibodies, anti-HIF-1 alpha antibody (Novus Biologicals) diluted to 1:500 and anti-β-tubulin antibody (MP Biomedicals, LLC) diluted to 1:2000. After the membranes were washed with TBS-T, they were incubated with secondary antibody (1:5000) for 1 h at room temperature. Antibody reaction was detected using an enhanced chemiluminescence system (GE Healthcare), and images were obtained by a multipurpose charge-coupled device (CCD) camera system (LAS 4000; Fujifilm Co.). The intensity of the reaction was measured by an analysis software application (Multi Gauge; Fujifilm Co.).

Immunocytochemistry

ASCs were cultured on noncoated glass coverslips at a density of 2 x 10⁵/mL. Cells were fixed with 10% neutral buffered formalin, treated with 0.1% (v/v) Triton X-100 in PBS for 10 min and incubated sequentially with Block Ace. They were incubated with monoclonal mouse Oct3/4 antibody (Neuromics) diluted at 1:200 for 1 h at room temperature. After washing, they were incubated with Alexa Fluor® 488 donkey anti-goat IgG (H+L) conjugate (1:200; Life Technologies Corporation) for 1 h and then 4’-diamino-2-phenylindole (DAPL; Pierce). Immunofluorescence images were recorded with an Axio Imager A2 microscope (Carl Zeiss Microscopy) equipped with an AxiosCam cooled-CCD camera system (Carl Zeiss Microscopy), which was operated with AxioVision software (Carl Zeiss Microscopy).

Reverse-transcriptase polymerase chain-reaction and quantitative real-time reverse-transcriptase polymerase chain reaction

cDNA was synthesized from total RNA using SuperScript III Reverse Transcriptase (Life Technologies Corp.). Polymerase-chain-reaction (PCR) amplification was performed with Taq DNA Polymerase (Life Technologies Corp.) using the Veriti™ Thermal Cycler (Life Technologies Corp.). Primer sets were prepared as in Table 1.

For real-time reverse-transcriptase (RT)-PCR, total RNA was isolated with Trizol® reagent (Life Technologies Corp.). cDNA was synthesized from 2 μg total RNA using SuperScript VILO® reagent (Life Technologies Corp.). The mRNA levels of various genes were analyzed by quantitative (q)RT-PCR using TaqMan® Gene Expression Assays (Applied Biosystems) with the 7500 Real-Time PCR System (Applied Biosystems). The RNA level of each target gene was normalized to β-actin as an internal control.
Characterization of ASCs

Within a few hours after the initial plating of the primary culture, ASCs attached to the dish bottom. They soon appeared with a spindle-shaped, fibroblastic morphology. We first evaluated cell surface markers associated with somatic stem cells. FACS analysis of ASCs at P2–3 demonstrated that the cells were positive for CD29 and CD44, similar to other stromal cells, but negative for CD117 (c-kit). Moreover, the cells were positive for Sca-1 similar to hematopoietic cells, but negative for CD117 (c-kit). Therefore, we assumed there was no significant difference in terms of cell viability. The growth curve revealed that both of their proliferative potentials decreased gradually as they grew larger passages. However, ASCs cultured at 2% O2 had 1.5 fold as much proliferative potency. This corresponded to 6.3 fold (4.70 × 10^11 vs. 7.52 × 10^10) over the 6 weeks of culture (Fig. 2c). We also evaluated the amount of HIF-1α protein to prove that cells were actually exposed to low oxygen (Fig. 2d). Cobalt (II) chloride exposure (100 µM) was used as the positive control.

Quantification of growth factors

ASCs (1 × 10^5 cells) were plated on 100-mm diameter cell culture dishes. ASC-conditioned medium was collected when cells became confluent. At the end of the culture period, supernatants were collected and stored at −80°C until analysis. The presence of VEGF and HGF in cell culture supernatants was measured using an enzyme-linked immunosorbent assay kit (RayBiotech Inc.). Control medium with 10% FBS was used as the control, and data were compensated with the ratio of cell number.

Differentiation assays

The ASCs between passages 1 and 5 were plated at 3 × 10^4 cells/cm² on culture dishes in the conditioned medium. After 24 h, medium was removed, and cells were rinsed twice with DPBS. Adipogenic and osteogenic differentiation were induced by culturing ASCs for 3 weeks in Aipogenic Differentiation Media (Cellular Engineering Technologies Inc.) and Osteogenic Differentiation Media (Cellular Engineering Technologies Inc.), respectively. Oil-red O and alkaline phosphatase (ALP) assays were examined with the Lipid Assay kit (Primary Cell Co.) and ALP Assay Kit (Primary Cell Co.) according to the procedures of the manufacturer. Quantification of the fatty drop was also performed with the Lipid Assay kit.

Statistical analyses

Results were expressed as means±SE. Statistical analyses were performed using the StatView 4.02J software package (Abacus Concepts). Statistical evaluations were compared using standard one-way analysis of variance followed by the Bonferroni post hoc test. A value of p < 0.05 was considered to be significant.

Results

Characterization of ASCs

Within a few hours after the initial plating of the primary culture, ASCs attached to the dish bottom. They soon appeared with a spindle-shaped, fibroblastic morphology. We first evaluated cell surface markers associated with somatic stem cells. FACS analysis of ASCs at P2–3 demonstrated that the cells were positive for CD29 and CD44, similar to other stromal stem cells, but negative for CD117 (c-kit). Moreover, the cells were positive for Sca-1 similar to hematopoietic cells, but only part of the cells expressed CD34 (Fig. 1a).

We next determined specific markers of stemness expressed in ASCs. RT-PCR detected mRNA expression of Nanog and Sox2 (Fig. 1b). Nanog, Sox2, and Oct3/4 are essential transcription factors for maintaining the pluripotent embryonic stem cell phenotype. Immunoreactive Oct3/4 was expressed strongly in the nuclei in the shape of spots, and shuttled between nuclei and cytoplasm at the time of cytokodieresis (Fig. 1c).

Effect of hypoxia on cell growth

Low oxygen tension has been shown to enhance the proliferation of multiple types of stem or progenitor cells. As shown in Figure 2, ASCs cultured at 2% O2 (Fig. 2b) exhibited higher cell growth at 21% O2 (Fig. 2a). To make the growth curve, only trypan blue negative viable cells were counted, and there was no significant difference in terms of cell viability. The growth curve revealed that both of their proliferative potentials decreased gradually as they grew larger passages. However, ASCs cultured at 2% O2 had 1.5 fold as much proliferative potency. This corresponded to 6.3 fold (4.70 × 10^11 vs. 7.52 × 10^10) over the 6 weeks of culture (Fig. 2c). We also evaluated the amount of HIF-1α protein to prove that cells were actually exposed to low oxygen (Fig. 2d). Cobalt (II) chloride exposure (100 µM) was used as the positive control.

Ability to differentiate

There was no morphological difference in the cells between hypoxia and normoxia. Oil-red–positive adipocytes were analyzed after adipogenic induction. Although the ratio of Oil-red–positive adipocytes was the same in the two groups at P1 (Fig. 3a), the adipogenic differentiation ability of ASCs was markedly decreased under the normoxic condition at P5 (~1 month of culture; Fig. 3b). In contrast, the hypoxic condition maintained a significantly higher ability for adipogenic differentiation. A similar phenomenon was detected in osteogenic differentiation. After osteogenic induction, ALP-positive osteocytes were markedly reduced under the normoxic condition at P5. However, under the hypoxic condition, the ability of osteogenic differentiation was sustained (Fig. 3d). Quantitative analysis of Oil-red revealed that hypoxia retained about twofold higher ability in terms of adipogenic differentiation at P5 (Fig. 3e). In terms of neurogenic differentiation, there were no significant changes at P5 under the normoxic condition. Therefore, we assumed there was no difference between normoxia and hypoxia (data not shown).
in Oct3/4 and Nanog expression for normoxia from P1 to P5, although the change was much higher for hypoxia.

We next investigated the amount of some growth factors in culture medium. Cultured ASCs secreted a significant amount of growth factors and cytokines such as HGF and VEGF. We found an increase in HGF secretion by five- to sixfold at P5 under both conditions. In contrast, VEGF secretion was significantly higher under hypoxia than normoxia only at P5 (Fig. 4b). Basic fibroblast growth factor and epidermal growth factor were not detectable in this study (data not shown).

Discussion

This study demonstrated that low oxygen tension enhances the ability of ASCs to maintain their differentiation potential. ASCs display multilineage developmental plasticity in vitro and in vivo. There are obvious similarities between ASCs and BMSCs (e.g., gene profiles, functions, and cell surface markers). Moreover, both cell types express CD29, CD44, and Sca-1 (Fig. 1a), which have been used to define mesenchymal stem and progenitor cells capable of adipogenesis, chondrogenesis, osteogenesis, and hematopoietic support. Although ASCs showed no expression of CD117 (c-kit) and only partial expression of CD34, they appeared to maintain several properties of self replication and differentiation into multiple cellular lineages, namely “stemness” (Fig. 1a).

The emergence of Oct3/4 in ASCs has been reported. However, little is known about the molecular mechanisms for the control of growth and differentiation of adult somatic stem cells including ASCs. It is important to understand how Oct3/4 expression is regulated in ASCs. Oct3/4 and Sox2 maintain pluripotency of ES cells, and they are also famous transcription factors in the production of iPS cells. The POU domain–containing Oct3/4 and the HMG domain–containing Sox2 are two other transcription factors known to be essential for normal pluripotent cell development and maintenance. In undifferentiated mouse and human ES cells, Oct3/4 and Sox2 also interact with the Nanog promoter. Nanog transcripts are directly regulated by Oct3/4 and Sox2 and maintain pluripotency of ES cells. These factors have been suggested as candidates for master regulation of initiation, maintenance, and differentiation of pluripotent cells, and our results indicate that they play important roles in differentiation potentials of ASCs. The microenvironment in various types of stem cells is important, and hypoxia plays a critical role in maintaining their characteristics. A relationship has been demonstrated between hypoxia, HIF, and certain transcription factors that are crucial for the regulation of stem cell differentiation. HIF plays a crucial role in its signal transduction and regulates
development or differentiation physiologically. In the present study, ASCs were shown to have a slower growth rate at higher passages under both normoxic and hypoxic conditions. Compared with normoxia, ASCs cultured at 2% O_2 had 1.5 fold as much proliferation potency over the 6 weeks of culture (Fig. 2c). Notably, the proliferation of ASCs is not linearly different from other stem cells. This may indicate that the degree of ASC maturation is a little higher than that of other stem cells.

We then investigated the differentiation potency of ASCs at P5 (after 1 month of culture). When ASCs were cultured under the normoxic condition, the ability of adipogenic differentiation was significantly decreased (Fig. 3a, 3b). On the other hand, the hypoxic condition maintained the adipogenic ability. Quantitative analysis of fatty drops revealed that hypoxia retained its adipogenic ability at almost the same level as that at P1 (Fig. 3e), which was similar to the findings for osteogenic differentiation (Fig. 3c, 3d). Since low oxygen tension itself has been reported to inhibit in vitro chondrogenesis and osteogenesis in ASCs, we tried to confirm whether hypoxia is able to maintain ASCs in their undifferentiated state without decreasing their differentiation potential. In the case of 3T3L1 preadipocytes, hypoxia maintained their undifferentiated phenotype. Thus, the adipogenic and osteogenic abilities of ASCs (at P5) cultured in hypoxic condition suggest that hypoxia in the microenvironment may be important for the maintenance of the multilineage differentiation potential because adipocytes belong to the mesoderm and osteocytes belong to the ectoderm. Interestingly, there was no significant difference in terms of neurogenic induction at P5 (data not shown). Therefore, it seems that the degree of loss in ability changes with the direction of differentiation.

FIG. 2. Cell growth analysis of ASCs in low oxygen tension. (a,b) Phase-contrast images of ASCs (passage 1) show morphology changes relative to the duration of the culture. Compared with the normoxic condition (a), the growth of ASCs was higher under the hypoxic condition (b). (c) The growth curve represents the difference in expansion potentials under each condition. Only trypan blue negative living cells are counted. Changes in the low oxygen concentration (●) and normal oxygen concentration (○) are noted. **p < 0.01 versus normoxia. (d) HIF-1 alpha protein was increased under the hypoxic condition. Cobalt (II) chloride (CoCl_2; 100 mM) exposure was used as the positive control.
ASCs maintained in the low oxygen tension for a period of 1 month showed an increase in pluripotency markers Oct3/4 and Nanog (Fig. 4a). Careful examination is needed regarding the increase of Oct3/4 and Nanog at P5 because it may not simply reflect the undifferentiated state since adipogenic and osteogenic potency at P5 decreased. Heterogeneity may explain this discrepancy. Immediately after the cell harvest (P1) ASCs are slightly contaminated with other cells, i.e., myofibroblasts or endothelial cells.32,33 These contaminating cells are gradually eliminated in subsequent passages, suggesting that simple comparison of P1 to P5 is inappropriate. On the other hand, cells in both experimental groups (normoxia and hypoxia) retained the same typical spindle-shaped morphology at P5, suggesting that comparison of hypoxia to normoxia is appropriate. Hypoxia maintains a higher level of Oct3/4 and Nanog at 1 month of culture (Fig. 4a). HIF-2α directly promotes Oct3/4 and regulates cell growth or differentiation;34 a similar phenomenon has been observed in terms of growth factor secretion. Although there is no difference for HGF, VEGF secretion is significantly higher in the hypoxic condition only at P5 (Fig. 4b). Hypoxia increases VEGF secretion in ASCs to stimulate vasculogenesis and angiogenesis.35 Secreted VEGF directly stimulates ASCs via VEGF receptors in an autocrine manner and regenerates neighboring cells in a paracrine manner,36 which could contribute to up-regulation of cell growth and proliferation.

In summary, low oxygen tension enhances proliferation and maintains stemness of ASCs. Cell cultures under low oxygen tension are beneficial to ASC proliferation and maintenance of their undifferentiated state for further therapeutic use.

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Author Disclosure Statement
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