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EFFICACY OF AUTOLOGOUS SERUM IN HUMAN ADIPOSE-DERIVED STEM CELLS; CELL MARKERS, GROWTH FACTORS AND DIFFERENTIATION

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Abstract

Human adipose-derived stem cells (hASCs) are a feasible source of stem cells for use in clinical applications. hASCs are typically cultured in medium containing fetal bovine serum (FBS); however, use of FBS is not recommended due to issues of clinical safety with regard to infections or immune response. Replacement of FBS with autologous human serum (autoHS) can eliminate these problems; however, their maintainability as potent ASCs in autoHS needs to be confirmed. Thus, we conducted an investigation of characterizations and functions of hASCs grown in medium containing autoHS compared to FBS. Cell counting and the WST-8 assay were used in assessment of the proliferation rate. In hASC cultured with culture medium plus autoHS or FBS, cell phenotypes were characterized by flow cytometry (CD13, CD29, CD31, CD34, and CD44) and expression of BDNF, HGF, IGF, LIF, NGF, and VEGF was determined by RT-PCR. Adipogenic differentiation was confirmed by oil red O stain. hASC showed greater expansion in AutoHS than in FBS. Cell surface markers of hASCs grown in autoHS (autoHS-hASCs) were similar to markers of those grown in FBS (FBS-hASCs). AutoHS-hASCs also expressed multiple growth factors as well as FBS-hASCs. In addition, autoHS was effective in growth of hASCs as well as FBS and autoHS-hASCs retained their ability for adipogenic differentiation. In summary, autoHS-hASCs have multiple growth factor expressions with the same cell surface markers as FBS-hASCs *in vitro*. Our results suggest that autoHS can provide sufficient *ex vivo* expansion of hASCs.

Key words: Autologous human serum, clinical application, fetal bovine serum, human adipose-derived stem cells.

Article information's

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Abbreviations: **alloHS**: allogenic human serum; **autoHS**: autologous human serum; **BMSCs**: bone marrow stem cells; **BDNF**: brain-derived neurotrophic factor; **FBS**: fetal bovine serum; **bFGF**: basic fibroblast growth factor; **GM-CSF**: granulocyte monocyte colony stimulating factor; **HGF**: hepatocyte growth factor; **hASCs**: Human adipose-derived stem cells; **IGF-1**: insulin-like growth factor-1; **LIF**: Leukemia Inhibitory Factor; **MSCs**: mesenchymal stem cells; **NGF**: Nerve growth factor; VEGF: vascular endothelial growth factor.

INTRODUCTION

Human adipose-derived stem cells (hASCs) are easily isolated from tissue remaining after liposuction and have the ability to differentiate into several lineages, including adipocyte, bone, cartilage, skeletal muscle, endothelium, hematopoietic cells, and neuronal cells (7, 27, 38). In addition, hASCs secrete multiple growth factors, including granulocyte monocyte colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) (24, 26), which are effective as a paracrine effect in cell therapy for neurodegenerative diseases (16). Therefore, hASCs offer a clinically feasible source of stem cells for use in cell-based therapy.

When isolated from adipose tissue, hASCs may not initially be sufficient in number; therefore, *ex vivo* expansion of hASCs is necessary prior to performance of clinical trials. Culture conditions for hASCs isolation and expansion require medium supplemented with fetal bovine serum (FBS) for generation of clinically relevant numbers of cells (26). However, addition of FBS includes the possibilities of viral or bacterial infection and the risk of prion, which can cause variant forms of Creutzfeldt-Jakob disease (12, 35). Immune or local inflammatory reactions due to contamination with bovine proteins is another potential problem associated with FBS (9, 19, 30, 33). Use of autologous human serum (autoHS), which also contains the growth factors and substances necessary for isolation and expansion, is one solution to the safety issues associated with FBS. Use of autoHS in primary culture of hASCs or bone marrow stem cells (BMSCs) for clinical application has been investigated (11, 20, 22, 29, 31); autoHS was found to be safer for use in clinical applications than other serums, such as animal-derived serum and allogenic human serum (alloHS) (5, 25, 31). However, the question of whether expansion of ASC in autoHS may cause changes to cell markers and growth factors has not been completely clarified with differentiation capacity (32).

In this study, we obtained ASCs and serum from the same volunteers, and cultured hASCs with autoHS. These hASCs are characterized by flow cytometry and RT-PCR in comparison with hASCs grown with FBS.

MATERIALS AND METHODS

Isolation and expansion of human ASCs

All procedures were approved by the institutional review board of Seoul National University Hospital. Subcutaneous adipose samples were acquired. Human adipose-derived stem cells were cultured as previously described (26). Subcutaneous adipose samples and blood were acquired with the consent of the donor, who is a 33-year old female. Subcutaneous adipose samples were digested in collagenase type 1 solution (Invitrogen, Carlsbad, CA, USA) with gentle agitation for 1 h at 37°C. Mature adipocyte fractions were separated from stromal fractions by centrifugation at 1200×g for 10 min. The remaining stromal fractions (pellet) were treated with red blood cell lysis buffer for 10 min at room temperature, filtered through a 100-μm nylon mesh, and centrifuged at 1200×g for 10 min. The pellet was resuspended and cultured in DMEM (Gibco-BRL) with 1 g/L glucose (Gibco-BRL), 1% penicillin/streptomycin (Gibco-BRL), and 10% heat inactivated FBS (Gibco-BRL) or 10% heat inactivated autoHS (see below) at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. After 1 day, non-adherent cells were washed out and plastic adherent hASCs were further expanded with the medium described above and fed every 3 days.

Autologous human serum

Fifty ml of whole blood was incubated for 2 h at room temperature, and centrifuged at 1,800×g at 4°C for 10 min. Serum was collected and filtered through a 0.2-μm membrane, aliquoted, and stored at -20°C until ready for use. The serum was heat inactivated prior to the experiment.

Preparation of extract from Adipose-derived stem cells

ASC's cytoplasmic extracts were prepared by suspension of cells in serum-free culture media, subjecting them to three cycles of rapid freeze/thawing and isolation of the supernatant after centrifugation at 15000 g. The BCA Protein Assay Kit (Rockford, USA) was used for quantification of the total protein content of the extract.

Measurement of cell proliferation

Cell counting and the WST-8 (Dojindo Laboratory) assay were used in investigation of cell proliferation. hASCs from passage 3 (P3) were seeded into a 6-well plate at 5×10^4 cells per well and counted every day by hemocytometer after detachment of cells with 0.25% trypsin-EDTA (Gibco-BRL). The WST-8 assay was used for investigation of cell proliferation, as described previously (1). In brief, hASCs from P3 were seeded into a 96-well plate (1×10^3 cells per well) according to the manufacturer's instructions. The plates were read on a Biotek EL-312e microplate reader (Biotek Instruments, Winooski, VT) at 48 h and 72 h after incubation, using a test wavelength of 450 nm and a reference wavelength of 650 nm. The mean and SD of each group were obtained. This experiment was performed in triplicate.

Flow-cytometric analysis for cell surface markers

Flow cytometry was performed on hASCs grown in 10% autoHS or 10% FBS. In brief, human ASCs from 3 passages were collected, washed with PBS, and stained with antibodies for 30 min in the dark. Antibodies were added in concentrations recommended by the manufacturer (20 μl/10^6 cells). Mouse anti-human antibodies were PE-, PE-cy5 or FITC-conjugated, and were purchased from BD Pharmingen: CD13 (IgG1, PE), CD29 (IgG1, PE-cy5), CD31 (IgG1, PE), CD34 (IgG1, FITC), CD44 (IgG1, FITC). hASCs from P3 were used for flow-cytometric analysis.

RNA preparation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

For qRT-PCR analysis, total RNA was isolated from hASCs grown in 10% autoHS or 10% FBS at passage 3 using the QIAshredder and RNeasy kits (Qiagen, Valencia, CA, USA). RNA samples were reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). The qRT-PCR analysis was validated for each mRNA, and primers for RT-PCR are as follows: BDNF (-TGA GCC TGT GTG ACA GTA TT-, -CAG CCT TCT TTT GTG TAA CC-), HGF (-AGA TTG TTA TCG TGG GAA GAA TG-, -GAG CAT CAT CAT CTG GAT TT), LIF (-AAC AAC CTC ATG AAC CAG AT-, -GTG CCA AGG TAC ACG ACT AT-), NGF (-GGA CGC AGC TTT CTA TCC TGG-, -CCC TCT GGG ACA TTG CTA TCT G-), VEGF (-GTG GAC ATC TTC CAG GAG TA-, -TCT GAC TTC ACA TTT GTT GT-). This experiment was performed in triplicate.

Adipogenesis assay

hASCs were harvested using trypsin/EDTA and plated in multiple-well plates at 1×10^4 cells/cm^2 for 24 h in 10% autoHS or 10% FBS containing DMEM (Day 0). On Day 1, the medium was then changed to adipogenesis differentiation medium (StemPro○,R Adipogenesis Differentiation Kit, Gibco invitrogen) for a 3-day period. From Day 4 onward, the cells were fed every 3 to 4 days. Differentiation was confirmed by oil red O stain as an indicator of intracellular lipid accumulation. Briefly, the cells were fixed for 5 min at room temperature in 10% formaldehyde and washed with 60% isopropanol. After the wells were completely dry, the cells were then incubated in 2% (w/v) oil red O reagent for 10 min at room temperature. Excess stain was removed by washing with 70% ethanol, followed by several changes of distilled water. All experiments were performed more than three times.

RESULTS

Isolation and proliferation of hASCs

Isolation and culture of ASCs from human adipose tissue was performed under both autoHS and FBS conditions. We observed the morphology of hASCs grown in 10% autoHS or 10% FBS during primary culture (PC) and passage 3 (P3). hASCs isolated (PC) and expanded (P3) in 10% autoHS were morphologically similar to those in 10% FBS (Fig 1A). To investigate growth of hASCs, we counted the number of cells cultured with 10% autoHS or 10% FBS every day and analyzed cell proliferation by the WST-8 assay. As shown in Figure 1B, proliferation of hASCs was faster in 10% autoHS than in 10% FBS and doubling time in log phage of growth was 15 h vs. 20 h, respectively. Proliferation of hASCs grown in 10% autoHS or 10% FBS was confirmed by the WST-8 assay at 48 h and 72 h after incubation. Compared with FBS, use of autoHS resulted in significant augmentation of the growth of cells (Fig 1C).

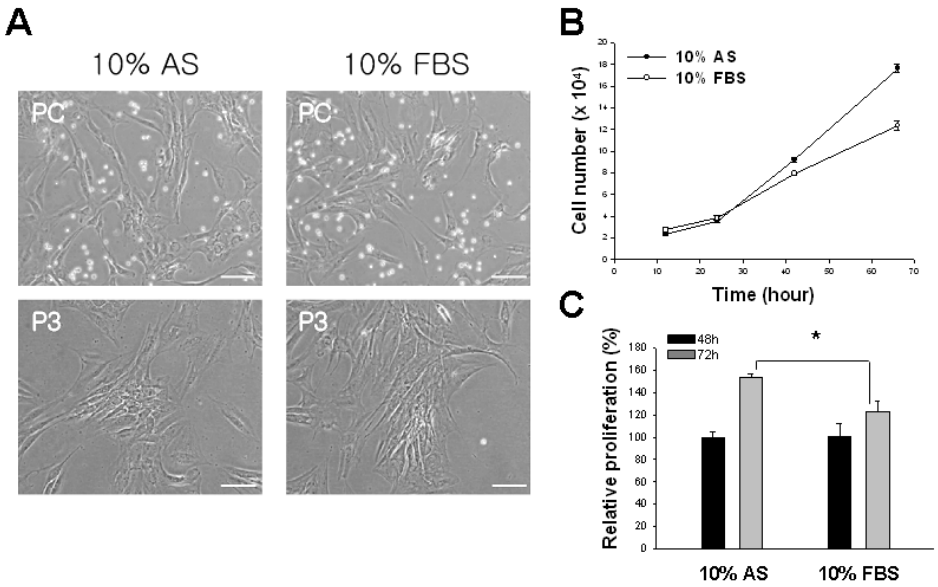

Figure 1. The morphology and proliferation of hASCs. (A) Cells on day 4 in PC (upper) and day 3 in P3 (lower) at 100× magnification. The morphological difference was not noted. (B) hASC (P3) grown in 10% autoHS or 10% FBS are counted at 12 h, 24 h, 42 h and 66 h. 10% autoHS shows a trend with higher number of cells in 42 h and 66 h.(C) The proliferation of hASCs was measured by WST-8 assay at 48 h and 72 h, showing significant difference in proliferation capacity. *P<0.001, Bar=100 μm

Surface phenotypes and growth factor expression in hASCs grown in autoHS or FBS

Biological characters of hASCs are similar to those of bone marrow-derived mesenchymal stem cells (MSCs) (6, 27, 34, 37). Both hASCs and MSCs express CD13, CD29, and CD44 cell-surface markers, but not hematopoietic marker CD34 nor the endothelial marker CD31 (6, 8, 28, 34). Flow cytometry analysis has shown that hASCs grown in 10% autoHS express CD13+, CD29+, CD31-, CD34-, and CD44+ cell-surface markers, as well as those in 10% FBS, showing CD13+, CD29+, CD31-, CD34-, and CD44+.

Growth factors secreted by ASCs include GM-CSF, VEGF, HGF, bFGF, and IGF-1, implicating a paracrine effect in cell therapy (16). We investigated mRNA levels of growth factors between these two conditions using hASCs

grown with ASC-extract-treated media as a control. qRT-PCR analysis indicated an increase of NGF mRNAs in autoHS, compared to FBS, and BDNF, HGF, and VEGF mRNAs showed similar expression (Fig 3). mRNA level of LIF decreased in autoHS, compared to FBS. Of particular interest, NGF was not almost expressed in hASCs grown with ASC-extract-treated media.

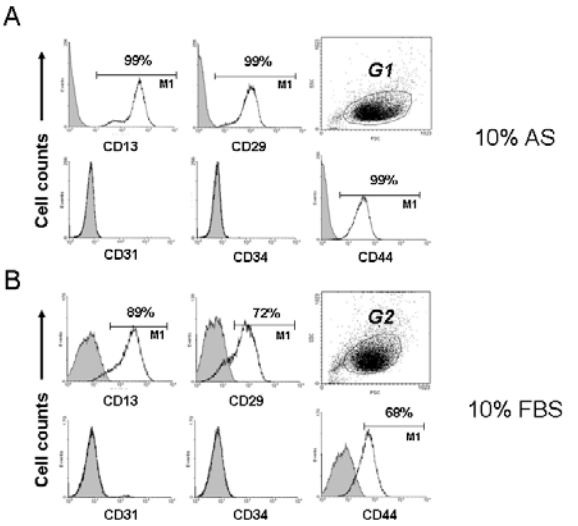

Figure 2. Flow cytometry analysis of cell surface markers on hASC. In P3, hASCs grown in 10% autoHS (A) or 10% FBS (B) were stained with a fluorescent-conjugated CD13, CD29, CD31, CD34 and CD44 and analyzed by flow cytometry. Positive staining was defined as a fluorescent intensity greater than 99% of control. ("M1" bracket in histogram panel). CD13, CD29 and CD44 are positive both in ASCs grown in autoHS and FBS whereas CD31, CD34 are negative. The patterns of cell surface markers between autoHS and FBS are similar. G1 and G2 mean gated-population 1 and 2 each group.

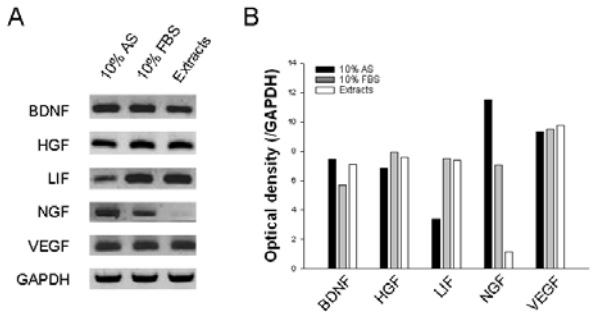

Figure 3. Analysis of mRNA levels in hASCs. 10% autoHS-hASCs (10% AS) shows higher levels of NGF mRNAs compared to those grown in 10% FBS-hASCs (10% FBS). BDNF, HGF and VEGF are similar whereas LIF is lower in 10% (A and B). NGF was not almost expressed in hASCs grown with ASC-extract-treated media (50 g/ml). GAPDH is used for control. (BDNF, brain-derived neurotrophic factor; HGF, hepatocyte growth factor; LIF, leukemia inhibitory factor; NGF, nerve growth factor; VEGF, vascular endothelial growth factor.).

Differentiation capacity of hASCs

hASCs have the capacity for differentiation into cells of mesenchymal origin, including adipocytes, myocytes, chondrocytes, and osteocytes (10, 14, 23, 37). To investigate the differentiation capacity of 10% autoHS-hASCs, these were induced into adipocytes by adipogenesis differentiation medium. After differentiation of hASCs into adipocytes, these cells were stained by oil red O. Figure 4 shows that hASCs grown in both 10% autoHS and 10% FBS differentiated into adipocytes. These results suggest that 10% autoHS-hASCs retained their potency, as shown by their adipogenic differentiation capability.

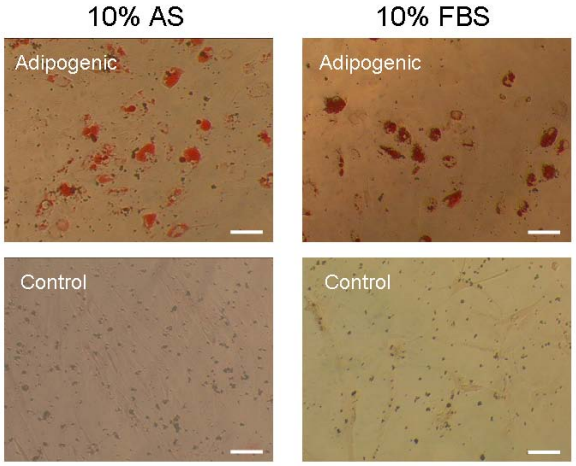

Figure 4. Oil red O staining of hASCs undergoing adipogenic differentiation. hASCs grown in 10% autoHS or 10% FBS were cultured for 10 days in adipogenesis differentiation medium (adipogenic) or growth media (control) and oil red O staining was performed. Bar=100 μm

DISCUSSION

In this study, we attempted to determine whether hASCs cultured with 10% autoHS shared similar properties with FBS, including appearance, growth expansion behavior, cell surface markers, expression of growth factors, and the ability for differentiation. Proliferation rate was higher with 10% autoHS than with 10% FBS. Cell surface markers of hASCs grown in autoHS were similar to those grown in FBS, showing CD13+, CD29+, CD31-, CD34-, and CD44+. mRNA expression of growth factors such as BDNF, HGF, IGF, LIF, NGF, and VEGF were noted in autoHS-hASC. AutoHS-hASCs retained their ability for adipogenic differentiation. Based on our data, we conclude that autoHS appears to be as good as FBS with regard to both isolation and expansion of hASCs.

hASCs are promising candidates for use in cell-based therapies. One major barrier to their

clinical use is the biosafety of FBS, which is a crucial part of all media currently used for culture of hASCs (26). To solve this problem, HS is used in generation of human BMSC and ASCs (5, 13, 21, 29, 32). Only a few reports have compared HS with FBS with respect to their growth stimulatory abilities (2, 3, 21, 36). The effects of HS on isolation and expansion of ASCs are controversial (15, 32). On the other hand, another group showed that a high concentration of HS is superior in differentiation capability of MSCs compared to a low concentration of HS (32). Allogeneic HS also provides sufficient *ex vivo* expansion of hASCs (3, 17). In our study, we tested autologous HS and found that 10% autoHS showed greater expansion than 10% FBS. We also isolated and expanded primary culture of hASCs using 1% or 5% autoHS. As a result, hASCs grown in 5% autoHS had a similar proliferation rate and differentiation to those grown in 10% autoHS, while 1% autoHS was inferior (data not shown).

We suggest that both 5% and 10% autoHS provide sufficient *ex vivo* expansion and isolation of hASCs possessing differentiation potentials.

In our culture of human ASCs, we had a limitation with regard to culture conditions for human cells, even though 5% CO^2 and 3% O^2 incubators are more appropriate for growth of hASCs than 5% CO^2 with 95% air condition (4).

In this study, flow cytometry characterization of cell surface markers in autoHS-cultured hASCs revealed similarities to those of FBS-cultured hASCs, suggesting that autoHS can maintain the phenotype of adipos-derived stem cells. In addition, FBS-cultured hASCs and adipos-derived stem cells are known to maintain multipotentiality under different culture conditions (18). We showed that autoHS-hASCS expressed mRNAs of multiple growth factors, including BDNF, HGF, IGF, LIF, NGF, and VEGF. However, the profile of these factor levels differed from those of FBS. In autoHS, when compared to FBS, increased expression of BDNF and NGF mRNAs resulted in decreased LIF, whereas HGF and VEGF mRNAs were similar. These results suggest to us that we consider the significance of the secretion pattern of growth factors associated with the paracrine effect in stem cell therapy.

In this study, adipogenesis was characterized by Oil Red O for proving the differentiation capacity in autoHS-cultured hASCs, indicating that autoHS-hASCS had a differentiation capacity that was at least similar to those grown in FBS. We did not perform an evaluation of the multipotency of hASCs in this experiment, which warrants further study, depending on different therapeutic strategies.

In conclusion, our results support that the use of autoHS provides a higher expansion rate than FBS, maintaining phenotype, differentiation potential as an ASC, and avoidance of contact with exogenous FBS as a source for cell therapy.

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