

Accelerated and Safe Proliferation of Human Adipose-derived Stem Cells in Medium Supplemented with Human Serum

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Abstract

Adipose-derived stem cells (ASCs) are a promising cell source and are being investigated for a variety of therapeutic applications. However, standard expansion protocols use fetal bovine serum (FBS) as a growth factor supplement, which is a potential source of undesirable xenogeneic pathogens. For clinical safety, autologous human serum (HS) would be more appropriate. This study compared FBS-supplemented and HS-supplemented media for their enhancement of the proliferation and differentiation potential of human ASCs (hASCs). HS was obtained from the blood of 8 healthy volunteers using collection devices specially designed to derive growth factors from platelets. Growth factors in HS or FBS were measured with enzyme-linked immunosorbent assays. The hASCs were isolated with an established protocol from discarded human fat tissues obtained during a medical procedure and cultured in a medium supplemented with either 10% HS or 10% FBS. The hASCs were collected at several time points for the proliferation assays. The capacity for differentiation into the osteogenic, chondrogenic and adipogenic lineages was assessed qualitatively with the histochemical stains von Kossa, Alcian blue, and Oil red O, respectively, and quantitatively with the qualitative reverse transcriptase polymerase chain reaction. Differences in cell surface marker expression between the HS-supplemented and FBS-supplemented cultures were examined with flow cytometric analysis. Proliferation assays showed that the growth of hASCs was more rapid in HS-supplemented medium than in FBS-supplemented medium. All cells grown in each medium expressed similar patterns of cell surface markers. The ASCs cultured in the HS-supplemented medium proliferated more rapidly than those cultured in the FBS-supplemented medium and retained their differentiation capacity and immunophenotype. These results support the establishment of a safe and rapid expansion protocol with autologous serum for cell-based therapies, such as tissue engineering and regenerative medicine, using hASCs.

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Key words: adipose-derived stem cells, human serum, mesenchymal stromal cells, fetal bovine serum, platelet-derived factors

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Introduction

Cell therapy has received much attention owing to the ability of stem/progenitor cells to regenerate damaged tissues and organs. To date, many cell types have been presented as promising candidates for regenerative cell therapy, including embryonic stem cells, induced pluripotent stem cells¹, and stem/progenitor cells from adult tissues². Despite the excellent potential of embryonic stem cells, there are many ethical issues concerning their use³. The technology to develop induced pluripotent stem cells may overcome these ethical issues without sacrificing multipotency. However, there are still some limitations to their practical use, such as cell regulation and genetic manipulation^{1,4,5}.

Mesenchymal stem cells are multipotent, nonhematopoietic stem cells that are typically obtained from bone marrow⁶ but can also be isolated from several other tissues, such as umbilical cord blood⁷ and adipose tissue⁸⁻¹¹.

Because subcutaneous adipose tissue is abundant and readily accessible¹², it is a promising source of stem cells. Despite these advantages, adipose-derived stem cells (ASCs) might need to be grown *in vitro*, depending on the circumstances. For example, in the case of cell-based therapy, ASCs must be cultivated and grown safely and rapidly, while their multipotency is maintained.

Common protocols for clinical trials and basic research employing human ASCs (hASCs) use a growth medium supplemented with fetal bovine serum (FBS)¹³. However, FBS might be an undesirable source of xenogeneic antigens and might transmit zoonoses, such as animal viruses or prions. Additionally, FBS has been implicated in anaphylactic or Arthus-like immune reactions in patients who received cells generated in an FBS-supplemented medium^{14,15} and can even lead to arrhythmias after cellular cardioplasty¹⁶. For clinical use, hASCs should ideally be prepared with autologous serum. Few reports have demonstrated how human serum (HS) affects hASC proliferation and differentiation¹⁷⁻²⁰.

Platelets provide many kinds of growth factors

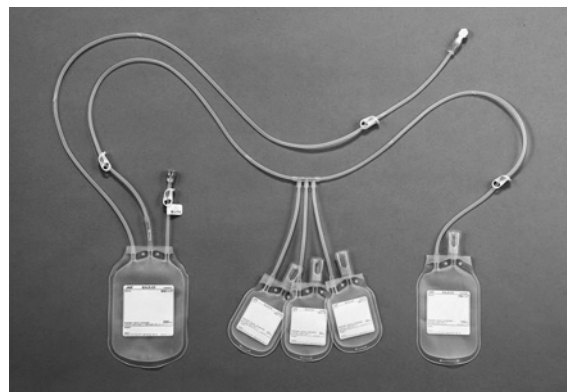


Fig. 1 Appearance of Cellaid®, a closed-system serum-collecting bag. The bags contain glass beads for platelet activation. The beads promote blood coagulation and release of growth factors.

that stimulate cell proliferation^{18,21,22}. A recently developed serum collection bag was specially designed to activate platelets and derive growth factors from them, and the efficacy of such preparations in stimulating cell proliferation has been confirmed with several cell types²³⁻²⁵. The aim of the present study was to investigate the efficacy of HS in preserving the proliferation and differentiation capacity of ASCs in cell culture.

Materials and Methods

Preparation of HS

Cellaid® (JMS Co., Ltd., Hiroshima, Japan), which is a completely closed serum-collecting bag system for separating serum for cell cultivation (**Fig. 1**), was used in these studies. The system is specially designed for collecting serum from whole blood, while activating platelets and removing fibrin. Two-hundred-milliliter samples of venous whole blood from 8 healthy donors (7 men and 1 woman; average age, 35.5 years; age range, 29 to 44 years) were collected in a large bag containing glass beads. The blood was then incubated at room temperature for 30 minutes with constant gentle agitation that allowed platelets to attach to the glass beads and release endogenous growth factors. Approximately 90 mL of HS from each donor was then separated with centrifugation at 2,500 rpm for 10 minutes. The serum was isolated, heat-inactivated at 56°C for 30

minutes, and stored at -80°C until use^{23,24}.

hASC Culture

Human subcutaneous fat was obtained from excess tissues excised during elective plastic surgery from 4 patients, with their written consent and that of their family. Tissue samples were used with the approval and according to the guidelines of the Institutional Review Board of Nippon Medical School based on the Declaration of Helsinki (Approval Number: 19-11-36). The hASCs were harvested and processed according to our established protocols²⁶⁻²⁸. Briefly, the fat tissue was excised and extensively washed with phosphate-buffered saline (Gibco-BRL, Grand Island, NY, USA). The tissue was then finely minced, digested in 0.15% collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and vigorously shaken for 30 minutes at 37°C in a 50-mL centrifuge tube. Next, an equal volume of either FBS-supplemented control medium (FBS-CM) containing Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL), 1% antibiotic-antimycotic (Gibco-BRL), and 10% FBS (Gibco-BRL) or HS-supplemented control medium (HS-CM) (DMEM, 1% antibiotic-antimycotic, and 10% HS) was added to neutralize the collagenase. The cell suspensions were then centrifuged at 1,300 rpm (260 g) for 5 minutes, after which the cell pellets were resuspended in each control medium. The ASCs were plated in 6-well tissue culture plates (2×10^5 cells/well) and maintained in each medium at 37°C in 5% CO_2 . The medium was replaced every 3 days. Once the hASCs had reached 80% to 90% confluence, they were detached from the culture dishes with 0.25% trypsin/EDTA (Gibco-BRL), neutralized with a control medium, and then passaged at a dilution of 1 : 3. To exclude differences due to lot-to-lot variability of FBS, 3 different lots of FBS were used.

Determination of Levels of Platelet-derived Growth Factor and Transforming Growth Factor- β in HS and FBS

The HS obtained with Cellaid[®] and the FBS were assayed to quantify the amounts of representative growth factors. Enzyme-linked immunosorbent

assays (ELISAs; all from R&D Systems, Inc., Minneapolis, MN, USA) for human transforming growth factor (TGF)- β 1 (Quantikine DB100), platelet-derived growth factor (PDGF)-AB (Quantikine DHD00B), and PDGF-BB (Quantikine DBB00) were performed for all samples of serum, HS (n=8), and FBS (n=3 different lots).

Cell Proliferation Assay

Second-passage hASCs were seeded at a density of 1.0×10^3 cells/well in 96-well culture plates. The cells were cultured with either FBS-CM or HS-CM and then collected at 2, 4, 6, 8, and 10 days after incubation. The numbers of cultured cells at each time point were measured with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions.

Flow Cytometric Analysis

At passage 6, the hASCs were detached with 0.25% trypsin/EDTA treatment. Aliquots of cell suspension containing 1×10^5 hASCs were incubated with antibodies dissolved in phosphate-buffered saline containing 3% FBS and 0.05% NaN_3 for 30 minutes on ice. Phycoerythrin-conjugated antibodies against the following cell surface markers were used: CD31, CD34, CD45, CD73, CD90 (BD Bioscience, Franklin Lakes, NJ, USA), and CD105 (eBioscience, Inc., San Diego, CA, USA). Flow cytometry was performed with a FACS-Calibur instrument (BD Japan, Tokyo, Japan) equipped with CellQuest software.

Differentiation Capacity of hASCs in HS-CM

Assays to induce differentiation into adipogenic, osteogenic, and chondrogenic lineages were carried out at the second cell passage.

For the adipogenic lineage, hASCs were seeded into 24-well culture plates at a density of 1×10^5 cells/well and cultured in either HS-CM or FBS-CM overnight. The cells were then incubated in DMEM supplemented with $1 \mu\text{M}$ dexamethasone (Sigma-Aldrich Co., St. Louis, MO, USA), $10 \mu\text{M}$ of insulin (Wako, Osaka), $200 \mu\text{M}$ of indomethacin (Sigma-Aldrich), and 0.5 mM isobutyl-methylxanthine

(Sigma-Aldrich), and either 10% HS or 10% FBS. The medium in each well was replaced twice a week. Two weeks later, the hASCs were fixed in 10% neutral buffered formalin for 25 minutes and stained with Oil red O.

For differentiation into the osteogenic lineage, hASCs were harvested and seeded at a density of 1×10^4 cells/well in 24-well plastic culture plates with either HS-CM or FBS-CM. The following day, the cells were incubated in DMEM supplemented with 0.1 μ M dexamethasone (Sigma-Aldrich), 50 μ g/mL ascorbic acid-2-phosphate (Sigma-Aldrich), and either 10% HS or 10% FBS. The medium was replaced twice per week. Three weeks later, mineral deposition was identified with von Kossa staining.

Chondrogenic differentiation was induced with the micromass culture technique²⁹. Briefly, 10 μ L of a concentrated hASC suspension (3×10^6 cells/mL) was placed in the center of each well of a 24-well culture plate and allowed to attach at 37°C in 5% CO₂ for 5 hours. Then, DMEM supplemented with 10 ng/mL TGF- β 1 (Sigma-Aldrich), 6.25 μ g/mL insulin (Sigma-Aldrich), 6.25 μ g/mL transferrin (Sigma-Aldrich), and either 10% HS or 10% FBS was gently overlaid without detaching the cell clusters, and the medium was changed every 3 days. The cultures were maintained for 3 weeks. Chondrogenesis was confirmed by the presence of proteoglycans identified with Alcian blue staining.

Quantitative Real Time-Polymerase Chain Reaction

Total RNA was extracted from cultures undergoing adipogenic, osteogenic, or chondrogenic differentiation using the Trizol reagent (Gibco-BRL) according to the manufacturer's protocol.

To quantify the expression level of a target gene in the trilineage differentiation at each time point, the quantitative real time-polymerase chain reaction (qRT-PCR) was performed with complementary DNA as a template. The genes of interest were those for peroxisomal proliferator-activated receptor (PPAR) γ for adipogenic differentiation, runt-related transcription factor (RUNX) 2 and sparc/osteonectin, cwcvc and kazal-like domains proteoglycan (SPOCK) 1 for osteogenic differentiation, Sry-related HMG box

(SOX) 9 for chondrogenic differentiation, and human 18S ribosomal RNA as a housekeeping gene (**Table I**). Total cellular RNA was isolated and reverse-transcribed with the TaqMan Gold RT-PCR kit for real-time PCR (Applied Biosystems, Carlsbad, CA USA). Quantitative RT-PCR was performed with this kit according to the manufacturer's instructions and an ABI 7700 Prism Sequence Detection system (Applied Biosystems, Carlsbad, CA USA). Analyses of the qRT-PCR were performed with SDS 1.2.3 software (Applied Biosystems, Carlsbad, CA USA). All data were normalized to the expression of the housekeeping gene (18SrRNA). The formula for calculating the relative messenger (m) RNA expression from the data was as follows:

$$\text{Relative mRNA expression: } 2^{-\Delta\Delta CT}$$

$$\Delta\Delta CT = CT_{\text{housekeeping gene}} - CT_{\text{gene of interest}}$$

CT is the threshold cycle (relative measure of the concentration of the target in the PCR reaction).

Statistical Analysis

The results were analyzed with *t*-tests (GraphPad Software, Inc., San Diego, CA, USA). All values are stated as mean \pm standard deviation. A *P* value of less than 0.05 was considered to indicate statistical significance.

Results

Levels of Growth Factors in Sera

Levels of the growth factors PDGF-AB, PDGF-BB, and TGF- β 1 in all HS (*n*=8) and FBS (*n*=3) samples were analyzed with ELISA. The concentration of PDGF-AB in HS (14.095 ± 5.310 ng/mL) was significantly greater (*p*<0.05) than that in FBS (0.239 ± 0.092 ng/mL). Similarly, the concentration of PDGF-BB was significantly greater (*p*<0.05) in HS (1.185 ± 0.2783 ng/mL) than in FBS (0.1840 ± 0.4808 ng/mL). Finally, the concentration of TGF- β 1 in HS (26.83 ± 2.083 ng/mL) was significantly greater (*p*<0.05) than that in FBS (11.18 ± 0.2791 ng/mL) (**Fig. 2 a**).

Proliferation of ASCs Was Accelerated in HS-CM

The ASCs grown in HS-CM showed more rapid

Table 1 List of primers used in qRT-PCR to identify expression of adipogenic, osteogenic, and chondrogenic differentiation factors

Gene		Primer 5'-Sequence-3'	Accession Number
PPAR γ	F	AGGAGCGGGTGAAGACTCAT	NM_015869
	R	CGAAGCTGCTCCAGAAAATG	
RUNX2	F	CCCGTGGCCTTCAAGGT	NM_0004348.3
	R	CGTTACCCGCCATGACAGTA	
SPOCK1	F	ACATCGGGCCTTGCAAATACA	NM_004598.3
	R	GAAGCAGCCGGCCCACTCATC	
SOX9	F	GCGGAGGAAGTCGGTGAAGA	NM_000346.3
	R	CCCTCTCGCTTCAGGTCAGC	
18SrRNA	F	GTAACCCGTTGAACCCCAT	NR_003286.2
	R	TCTTGGCAGCAGGATAGTCCTT	

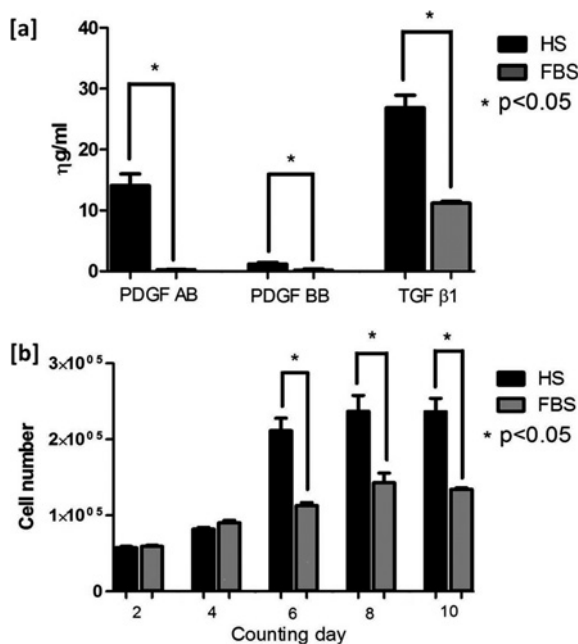


Fig. 2 Growth factor quantification in HS and FBS with ELISA and the proliferation potential of hASCs cultured in HS-CM and FBS-CM. [a] Concentrations of growth factors in HS (n=8) and FBS (n=3). The levels of PDGF-AB, PDGF-BB, and TGF- β 1 were significantly higher in HS than in FBS. [b] Cell numbers in each culture condition were counted on days 0, 2, 4, 6, 8, and 10. The cell numbers were significantly higher in HS-CM culture than in FBS-CM culture on days 6, 8, and 10 ($p < 0.05$).

proliferation than did ASCs grown in FBS-CM (**Fig. 2b**). The differences between HS and FBS were not significant on day 2 of culture ($57,304 \pm 1,828$ cells vs. $59,018 \pm 1,657$ cells, respectively; $p > 0.05$) or day 4 of culture ($81,872 \pm 2,198$ cells vs. $90,147 \pm 3,173$ cells; $p > 0.05$). However, the numbers of cells in HS-CM

were significantly greater than in FBS-CM on day 6 ($211,277 \pm 16,556$ cells vs. $112,885 \pm 4,108$ cells, respectively; $p < 0.05$), day 8 ($236,819 \pm 21,096$ cells vs. $142,819 \pm 12,924$ cells; $p < 0.05$), and day 10 ($236,293 \pm 17,800$ cells vs. $134,426 \pm 1,647$ cells; $p < 0.05$).

Culture in HS-CM Did Not Alter Cell Surface Markers of ASCs as Detected with FACS Analysis

The hASCs cultured in either HS-CM or FBS-CM showed identical patterns of surface protein expression with no significant variation in surface marker expression among different samples. All samples were positive for the surface proteins CD73, CD90, and CD105 and were negative for CD31, CD34, and CD45 (**Fig. 3**).

Differentiation Capacities of ASCs

At the second passage, hASCs cultured in medium supplemented with either HS or FBS were examined for their potential to differentiate into adipogenic, osteogenic, and chondrogenic lineages. A significant fraction of the cells cultured in adipogenic differentiation conditions contained multiple, intracellular lipid-filled droplets, as indicated by Oil red O staining. In all the osteogenically induced cells, calcium deposits were detected with von Kossa staining. All the cells induced to undergo chondrogenic differentiation exhibited pericellular proteoglycan deposition, as indicated by Alcian blue staining (**Fig. 4a**).

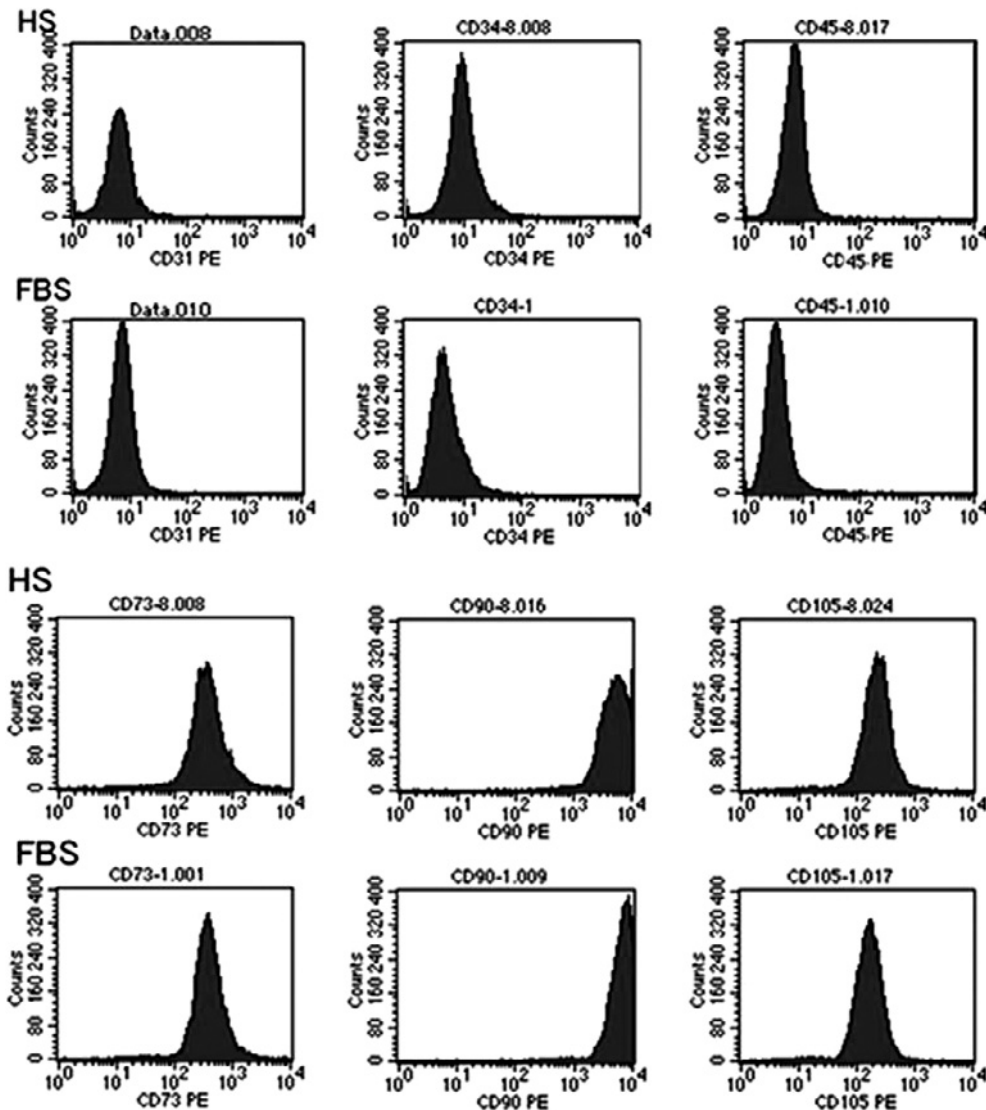


Fig. 3 Representative histograms of flow cytometric analysis of cell surface marker expression. Expression of CD31, CD34, CD45, CD73, CD90, and CD105 was measured in hASCs cultured in HS-CM or FBS-CM at passage 6. Both cells cultured in HS-CM and cells cultured in FBS-CM cultured showed similar patterns of expression: CD31 (-), CD34 (-), CD45 (-), CD73 (+), CD90 (+), and CD105 (+).

HS-supplemented Medium Induced Higher Levels of Gene Expression during Differentiation than FBS as Detected by qRT-PCR

After 2 weeks of adipogenic induction, the expression of PPAR γ was upregulated both in cells cultured in HS and in cells cultured in FBS, but no significant difference between HS (1.715 ± 0.2550) and FBS (1.235 ± 0.0350 , $p > 0.05$) was detected. Expression of 2 osteogenesis-specific genes (RUNX2 and SPOCK1) was analyzed with qRT-PCR after 28 days of osteogenic induction. The expression of RUNX2 and SPOCK1 was upregulated in cells from both HS-supplemented and FBS-supplemented

media, and RUNX2 showed significantly higher expression in HS-supplemented medium (2.08 ± 0.03) than in FBS-supplemented medium (0.98 ± 0.185 , $p < 0.05$). Chondrogenesis of ASCs cultured in either HS or FBS was analyzed by quantifying the expression of SOX9. The SOX9 expression after 3 weeks in induction medium was significantly higher in HS-supplemented medium (11.18 ± 0.5650) than in FBS-supplemented medium (1.275 ± 0.2750 , $p < 0.05$) (Fig. 4b).

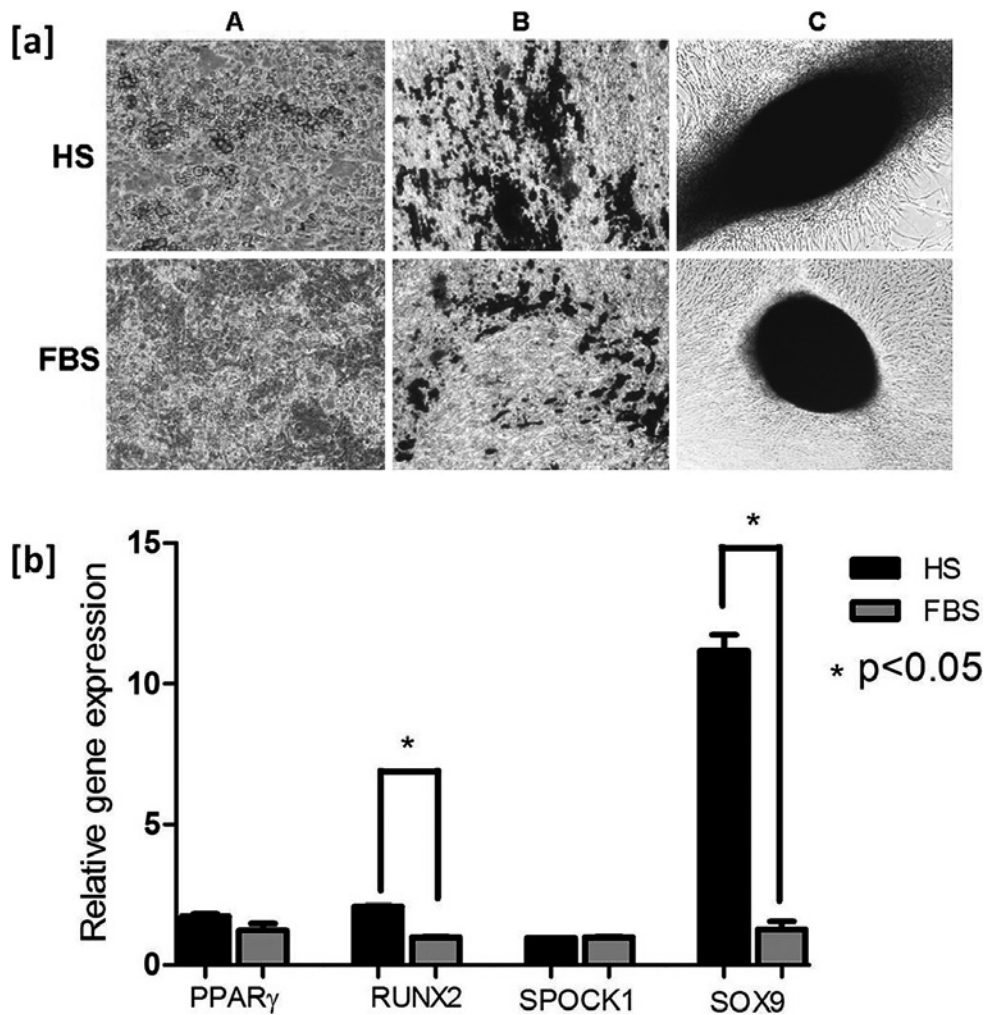


Fig. 4 Comparison of differentiation potential between hASCs cultured in differentiation medium supplemented with HS or FBS. [a] Microscopic results of cell differentiation: (A) adipogenesis (Oil red O staining), (B) osteogenesis (von Kossa staining), and (C) chondrogenesis (Alcian blue staining). Upper panel: cells cultured in HS-supplemented medium. Lower panel: cells cultured in FBS-supplemented medium. All cell populations in both media had similar patterns of differentiation. [b] Adipogenic, osteogenic, and chondrogenic gene expression in ASCs cultured in HS-supplemented induction medium compared with that in FBS-supplemented induction medium. Quantitative RT-PCR showed significantly higher expression of RUNX2 and SOX9 in HS-supplemented osteogenic and chondrogenic induction media ($p < 0.05$).

Discussion

A variety of on-going preclinical trials are using hASCs to search for treatments for such conditions as myocardial infarction³⁰⁻³² and liver failure³³. However, most isolation and expansion protocols for clinical-scale production of ASCs use culture medium supplemented with FBS, which contains xenogeneic proteins that can be internalized by ASCs³⁴. Consequently, a host of potential problems, such as viral and prion transmission and immunological

reactions, can arise^{14,35,36}.

The present results show that cell proliferation was more favorable with HS-CM than with FBS-CM. Sera from 8 different donors all showed favorable results, suggesting that autologous serum would produce similar effects. The accelerated expansion protocol would be highly valuable when the original amount of hASCs is limited, such as cell-based therapy with banked hASCs.

The surface marker expression of hASCs grown with HS-CM was indistinguishable from those cultured with FBS-CM. The expression patterns

after the sixth passage are similar to those reported previously³⁷. These results suggest that HS-CM did not differ from FBS-CM in its effects on differentiation, as evaluated with cell surface markers. Additionally, cells grown in HS were able to differentiate into 3 different lineages—adipogenic, osteogenic, and chondrogenic—like cells grown with FBS. Interestingly, our results revealed that osteogenesis and chondrogenesis showed a significantly higher expression of RUNX2 and SOX9, respectively, suggesting that hASCs cultured in HS have greater differentiation potential for osteogenesis and chondrogenesis than do hASCs cultured in FBS. These results indicate that HS has no adverse effects on the characteristic features or multipotency of hASCs. Taken together, these results suggest HS is more suitable for growing hASCs for clinical applications than is the commonly used conventional methods using FBS.

PDGFs are composed of 2 polypeptide chains (A and B) combined in 3 disulfide-linked dimeric forms (AA, AB, and BB)³⁸. Human platelets have all 3 PDGF dimers, in quantities of approximately 65% AB, 23% BB, and 12% AA. In this study, the concentrations of PDGF-AB and PDGF-BB in HS were evaluated. A previous report has indicated an optimal concentration of PDGF for proliferation of hASCs²¹. The ranges of PDGF-AB and PDGF-BB in HS obtained with Cellaid[®] in the present study were 7.5 to 24 ng/mL and 0.2 to 2.4 ng/mL, respectively. Therefore, the concentrations of growth factors in HS-supplemented medium were within the optimal concentration ranges. The proliferation of hASCs and the concentration of these growth factors showed a positive correlation and suggest that the levels of these growth factors have significant effects on ASC proliferation.

The Cellaid[®] serum collection bag system used in the present study contained glass beads that function as platelet activators. A gentle agitating process over 30 minutes allows the glass beads to remove fibrin from whole blood and to activate platelets. The levels of PDGF-AB, PDGF-BB, and TGF- β 1 were increased in all sera obtained, compared with those with standard HS collection methods. Because the Cellaid[®] bag is designed to

obtain serum in a completely closed system, the risk of microbial contamination is minimal. The usage of Cellaid[®] would make it possible to prepare autologous serum containing large amounts of cellular growth factors for cell-based therapy in the future.

Conclusions

This study has demonstrated that hASCs can be grown rapidly while preserving their multipotency with an HS-supplemented medium. The growth factors derived from platelets in HS enhanced cell proliferation. These results indicate that HS is a suitable substitute for FBS and can greatly improve the progress of basic research and cell-based therapy.

Conflict of interest: The authors have no financial conflicts of interest regarding the publication of this article.

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