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Human Serum Is an Advantageous Supplement for Human Dermal Fibroblast Expansion: Clinical Implications for Tissue Engineering of Skin

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Background. Standard fibroblast culture medium usually contains fetal bovine serum (FBS). In theory, unknown risks of infection from bovine disease or immune reaction to foreign proteins may occur if standard culture method is used for future human tissue-engineering development. Human serum (HS) theoretically would be another choice in providing a safer approach and autologous clinically reliable cells.

Methods. Isolated human dermal fibroblasts were culture-expanded in an equal volume mixture of Ham's F12 medium and Dulbecco's Modified Eagle Medium (DMEM) supplemented with either 10% HS or 10% FBS from passage 0 to passage 3. Effects of 10% HS and 10% FBS on human fibroblast viability, growth kinetics, cell cycle analysis and gene expressions were investigated and compared.

Results. Generally, fibroblast viability cultured in HS supplementations was much higher compared to FBS supplementation. Fibroblast proliferations were faster in HS supplementations with shorter doubling time. Cell cycle analysis showed fibroblasts cultured with HS supplementations have higher S-phase ratio compared to FBS. Gene expression levels by quantitative reverse transcriptase-polymerose chain reaction (RT-PCR) showed cultured fibroblasts with HS supplementation maintains expression of collagen type I collagen, increased expression of type III collagen and fibronectin and reduced expression of α-smooth muscle actin (α-SMA) compared to FBS.

Conclusions. Results demonstrated potential advantages of HS vs. FBS in generating larger numbers of cultured dermal fibroblasts in a shorter period of time. HS also influenced mRNA expression of type III collagen and fibronectin (upregulated) and α-SMA (downregulated), which are important extracellular matrix proteins in wound healing.

Key Words: Dermis, Fibroblast, Cell culture, Serum, Cell cycle, Gene expression.

Introduction

Tissue-engineered skin provides new approaches for the treatment of various skin injuries and disorders such as burns (1,2), diabetic ulcer, venous leg ulcer (3), bedsores and limb amputations (4). As huge amounts of fibroblasts are needed for tissue engineering of skin, the need to have high cell numbers within a short time when performing cell expansion is vital. The importance of fibroblasts has long been known, especially in skin tissue regeneration where they participate actively in wound healing processes. Fibroblasts synthesized various growth factors such as keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), insulin growth factor (IGF) and cytokines [(interleukin 8 (IL-8), interleukin 6 (IL-6))] that are important in stimulating wound healing processes (5,6). They also regulate epidermal

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differentiation processes, dermal regeneration and matrix deposition such as type I collagen, type III collagen, type IV collagen, elastin and laminin, thus important in accelerating the wound healing processes (5,7−10).

For the treatment of large skin defects, a higher number of viable dermal fibroblasts and keratinocytes are needed. Use of a medium such as Dulbecco's Modified Eagle Medium (DMEM) or combination medium of DMEM and F12 Ham's at various ratios (1:1, 3:1) with supplementation of fetal bovine serum (FBS) is common in the fibroblasts culture (5,10). FBS is a common supplement for *in vitro* cell, tissue and organ cultures. Supplementation of FBS is very important because the serum contains various growth factors, cytokines and proteins that will increase fibroblast proliferation (11−13). It also contains essential components such as hormones and vitamins. The composition of FBS varies among batches and may be contaminated with viruses, mycoplasms and prions (14). A previous study suggested the use of FBS may have the possibility of minimal risks because animal (bovine)-derived serum carries the remote possibility of transmission of prions or viral disease and zoonoses contamination (15). Several earlier studies in skin tissue culture reported immune response by antibody detection against bovine serum proteins in burn patients receiving keratinocytes graft cultured using FBS (16,17). Johnson et al. (18) showed that the persistence of bovine serum proteins in the skin graft is important in the induction of immune reaction causing human keratinocyte graft rejection.

Thus, in ensuring the safety of the graft and reducing the possibilities of immune reactions, human serum (HS) would probably be another choice in providing a safer approach to cultured fibroblast and for the formation of tissue-engineered skin. Previous studies of human cell culture from human osteoblasts (19), human bone marrow cells (20), chondrocytes (21−27), human conjunctival epithelial cells (28) and human cancellous bone-derived cells (29) showed autologous or pooled HS could promote higher cell proliferation compared to FBS. In the tissue-engineered skin formations (30), autologous HS will be useful in preventing the risk of immune reactions and late graft rejections.

The objective of this study is to evaluate and compare the effects of 10% HS and 10% FBS on human dermal fibroblast monolayer culture expansion. In this study the gene expression levels of types I and III collagen, fibronectin and α-smooth muscle actin (α-SMA-2) from culture-expanded dermal fibroblasts supplemented with 10% HS or 10% FBS were evaluated. Type I and type III collagen provide tensile strength to the skin with type I collagen as the most abundant collagen in the matrix of adult dermis tissues. Type III collagen acts as a major collagen in the early phase of wound healing, important in maintaining physiological functions and is the second most abundant collagen in human tissues (7). Fibronectin functions as the major component of granulation tissue during wound healing and in the migrations and attachments of cells, particularly fibroblasts, to the extracellular matrix (7,31). It is distributed on the cell surface and in plasma. α-SMA is a phenotype for myofibroblasts, dermal fibroblasts that change its gene expressions and become contractile to migrate to the wound site and facilitate in wound healing, thus involved in wound contracture (32,33).

Materials and Methods

This study was approved by the Universiti Kebangsaan Malaysia Research and Ethical Committee.

Isolation of Epidermal and Dermal Tissue from Full Thickness Skin

Skin samples were obtained from six consenting patients as redundant tissue from abdominoplasty (age range: 20−40 years). The skin was rinsed in 70% isopropanol and then placed into Dulbecco's Phosphate Buffered Saline (DPBS, Gibco/BRL, Grand Island, NY) containing 20 μg/mL gentamycin (Gibco/BRL) for 1 h. Skin was cut into small pieces (1−2 cm^2) and soaked in 25 caseinolytic units/ml (prepared from 1.10 caseinolytic units/mg Dispase stock) solution of Dispase (Sigma-Aldrich, St. Louis, MO) in defined keratinocyte−serum-free medium (DKSFM, Gibco/BRL) with 5 μg/mL gentamycin for 8−12 h at 2−8°C to separate the epidermis and dermis.

Extraction of Human Serum (HS) From Donor for HS Supplementation

Four hundred mL of fresh blood was withdrawn from a single donor (age 32 years) using serum-collecting tube with clot activator (Greiner Bio-One GmbH, Kremsmünster, Austria). The donor had undergone medical examination and was free from infectious diseases such as hepatitis and HIV, inheritable diseases and medical complications such as diabetes mellitus. Whole blood was kept at room temperature until clot formation was complete. Serum was then collected by centrifugation at 600 × g for 5 min at room temperature (25°C) and sterile-filtered using a 0.2-μm syringe filter (Sartorius, Edgewood, NY) yielding ~200 mL of HS. Serum was kept frozen at −20°C prior to use.

Dermal Fibroblast Cell Isolation and Cell Culture

Dermal fibroblast culture medium was prepared in one batch and divided into two groups. It was prepared using a combination of F12 Ham's/Dulbecco's Modified Eagle Medium (1:1) (F:D) supplemented with 0.1 % vitamin C, 0.1% glutamine, 0.1% Hepes buffer and 0.1% antibiotic−antimycotic (Invitrogen, Carlsbad, CA). One part of F:D medium was supplemented with 10% fetal bovine serum (FBS, Invitrogen) from a single batch (lot #41F7063 K) and one part F:D with 10% HS. Using a single batch of FBS and HS is very important to avoid variability in the results of both dermal fibroblast cultures.

Dermis layer was placed in 50 mL centrifuged tubes (Becton Dickinson, Rutherford, NJ) and digested with 0.6% collagenase type I enzyme (Gibco/BRL) for 12−18 h in an incubator shaker. The cell suspension obtained was centrifuged at 600 × g for 5 min at room temperature. The resulting pellet was washed with DPBS buffer and later resuspended in either F:D (1:1) + 10% FBS or F:D (1:1) + 10% HS depending on type of medium of the dermal fibroblasts cultured. Cells were stained using 0.4% trypan blue vital dye (Gibco/BRL) to distinguished dead and living cells and counted using a hemacytometer (Weber Scientific Int. Ltd., Lancing, England) to obtain total cells and viability number. Skin sample fibroblast was cultured individually (one sample for one set of experiment) in a six-well plate (Becton Dickinson) at the density of 1×10^5 cell per well separately in F:D (1:1) + 10% FBS and F:D (1:1) + 10% HS. Cells were cultured at 37°C in 5% CO_2 (Jouan, Nantes, France) with medium changed every 2−3 days. Fibroblast monolayer culture was subcultured from passage zero to passage three (P0−P3) when the culture reached confluence (~90% dermal fibroblast grown when observed under inverted microscope) (Leica, Wetzlar, Germany). Growth kinetics data of fibroblasts culture were observed, calculated and analyzed.

Cell Cycle Analysis Using Flow Cytometry

Two fibroblast passage 1 (P1) samples each cultured separately in F:D (1:1) + 10% FBS and F:D (1:1) + 10% HS were chosen for cell cycle analysis. Fibroblast from P1 culture was chosen because it provided the highest doubling time (DT) compared to other passages (P0, P2 and P3). A total number of 500,000 cells per sample were used. The fibroblasts were processed using CycleTEST PLUS DNA Reagent Kit (Becton Dickinson) following manufacturers' instructions. Using this kit, the single nuclei was isolated for the fibroblasts and stained with propidium iodide (PI). PI-stained single nuclei suspensions were analyzed using FACScan flowcytometers (Becton Dickinson) and raw data were collected using CELLQuest software (Becton Dickinson). A minimum of 50,000 cells was analyzed for one cycle of test for one sample and was repeated six times for each sample. Data analysis was performed using Modfit Cell Cycle Analysis Software (Verity House Software, Topsham, ME).

Total RNA Extraction for Quantitative Gene Expression Analysis

Total RNA from cultured fibroblasts in FBS and HS from every passage were extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Polyacryl carrier (Molecular Research Center) was added in each extraction to precipitate the total RNA. Extracted RNA pellet was then washed with 75% ethanol and dried before dissolved in Rnase- and Dnase-free distilled water (Invitrogen). Yield and purity of the extracted RNA was determined by spectrophotometer (Bio-Rad, Hercules, CA). Total RNA was stored at −80°C immediately after extraction.

Quantitative Gene Expression Analysis by Real-Time PCR

Gene expression for fibroblast extracellular matrix component (type I collagen, type III collagen, fibronectin and α-SMA-2) was quantitatively analyzed with real-time PCR technique. Keratin 14 is the marker for keratinocyte basal cells in the epidermal basal layer and was used to detect presence of keratinocyte contamination in the fibroblast culture. Expression level of each targeted gene was normalized to GAPDH. All primers (Table 1) were designed with Primer 3 on-line software and blasted with GenBank database sequences in order to obtain primers with high specificity. The efficiency and specificity of each primer set was confirmed with standard curve (Ct value vs. serial dilution of total RNA) and melting profile evaluation. Primer sequences used in this study are shown in Table 1. Real-time PCR reaction was performed with 100 ng of total RNA, 400 nM of each primer and iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) according to the manufacturer's instruction. Reactions were run using Bio-Rad iCycler with reaction profile of cDNA synthesis for 30 min at 50°C; pre-denaturation for 2 min at 94°C; PCR amplification for 38 cycles with 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. This series of cycles was followed by a melt curve

Table 1. Primer sequences used in real-time PCR for quantitative gene expression analysis[a]

Genes	Access #	Primer 5′-3′	PCR product (bp)
GAPDH	BC020308	F: 5′-tcc ctg agc tga acg gga ag-3′ R: 5′-gga gga gtg ggt gtc gct gt-3′	217
Type I collagen	NM 000088	F: 5′-agg gct cca acg aga tcg aga tcc g-3′ R: 5′-tac agg aag cag aca ggg cca acg-3′	222
Type III collagen	NM 000090	F: 5′-gtt gac cct aac caa gga tgc a-3′ R: 5′-gga agt tca gga ttg ccg tag-3′	203
Fibronectin	M10905	F: 5′-aag acc agc aga ggc ata agg-3′ R: 5′-cca ctt cca aag cct aag cac-3′	196
α-SMA-2	NM 001613	F: 5′-tgg ccg aga tct cac tga cta-3′ R: 5′-ctt ctc aag gga gga tga gga-3′	172
Keratin 14	BC002690	F: 5′-aga acc gca agg atg ccg ag-3′ R: 5′-act gca gct caa tct cca gg-3′	150

[a]Data from GenBank database sequence.

analysis to check reaction specificity. Expression level of each targeted gene was normalized to GAPDH and was then calculated for statistical analysis.

Statistical Analysis

Data for fibroblast viability and DT in each medium at every passage (P0, P1, P2 and P3) were collected from six samples. Values were presented as mean (±0.05) standard error of mean (SEM). Statistical analysis was performed using SPSS v. 12.0 for Windows. Differences in proliferation were assessed by comparing average number of DT value of HS and FBS individual serum supplementations at each individual passage. Growth kinetics (DT) of cultured fibroblasts in each media supplementation of HS and FBS at every passage (P0, P1, P2 and P3) was calculated for statistical analysis. The population DT is defined as average cell DT (days) in the culture.

Number of cell doublings:

$$Log\frac{Number\ of\ cells\ at\ end\ of\ culture}{Number\ of\ cells\ at\ start\ of\ culture}\times\frac{1}{Log2}$$

To identify an overall significant effect, analysis of variance (ANOVA) statistical analysis with Tukey honestly significant difference (HSD) method was used to compare individual groups. An α level of 0.05 was used to determine significance.

Data for fibroblast gene expressions of types I and III collagen, fibronectin, α-SMA-2 and type 14 keratin relative to the gene expression value of housekeeping gene GAPDH in each medium at every passage (P0, P1, P2 and P3) were collected from six samples. Type 14 keratin was used as a control to detect any contamination of keratinocyte cells in the dermal fibroblast cultures. Values were presented as mean (±0.05) SEM. Student's *t*-test was used to compare data between groups. To identify an overall significant effect, ANOVA with Tukey's HSD method was used to compare individual groups. An α level of 0.05 was used to determine significance.

Results

Morphology of Dermal Fibroblasts

Observation under inverted microscope showed fibroblasts (P0) cultured in both F:D + 10% FBS and F:D + 10% HS started to proliferate on the second day of culture and reached confluence within 8–12 days of monolayer culture. Cells became more elongated with increasing number of passages and 10% HS supplementation is much better because it increase attachments and proliferation of fibroblasts compared to FBS supplementation (Figure 1).

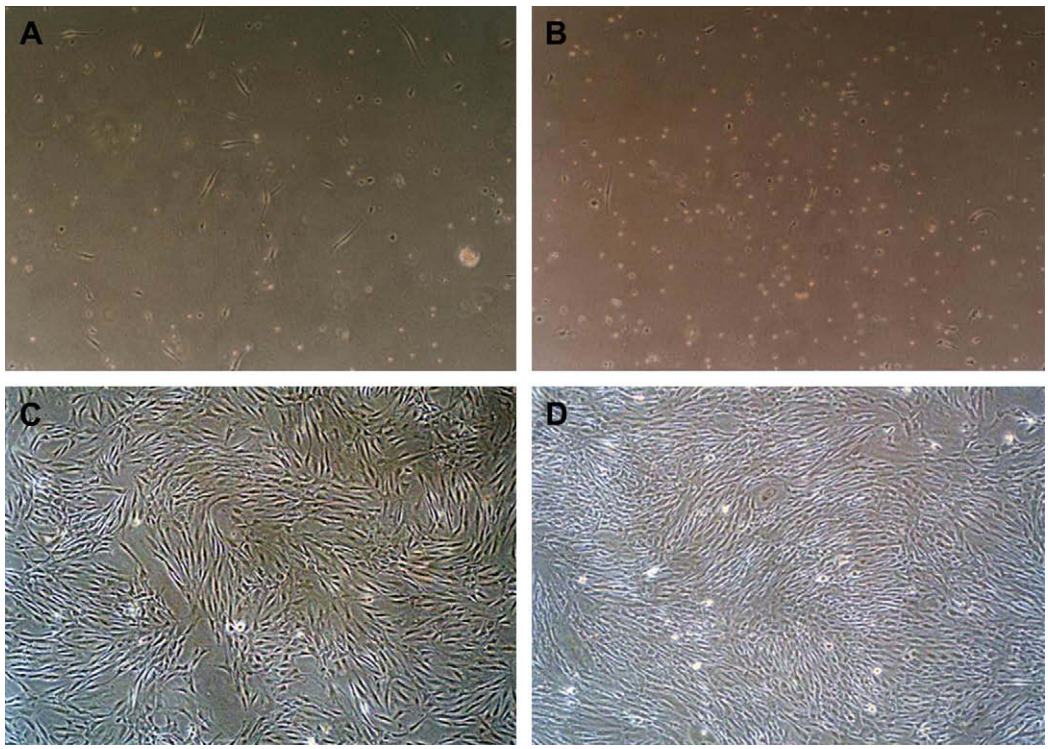

Figure 1. Dermal fibroblast culture, passage 0 (P0), 4 days after initial culture in F12 Ham's/Dulbecco's Modified Eagle Medium (1:1) (F:D) 10% fetal bovine serum (FBS) (×40) (A). Dermal fibroblast culture, P0, 4 days after initial culture in F:D 10% human serum (HS) (×40) (B). Dermal fibroblast culture, P0, confluence at 12 days of culture in F:D 10% FBS (×40) (C). Dermal fibroblast culture, P0, confluence at 12 days of culture in F:D 10% HS (×40) (D). Gross morphology showed dermal fibroblast cultured in supplementation of pooled 10% HS are more confluent showing higher culture-expanding capability. Color version of this figure available online at www.arcmedres.com

Growth Kinetics of Dermal Fibroblasts

Viability of fibroblasts showed the same pattern during culture at P0, P2 and P3 where fibroblasts cultured in F:D 10% HS have higher viability compared to cells cultured in F:D 10% FBS. The viability obtained at P0, P2 and P3 showed no significant differences. However, fibroblasts cultured at P1 in F:D 10% HS demonstrated significantly lower viability ($89.67 \pm 2.5\%$) compared to cells cultured in F:D 10% FBS ($96.67 \pm 0.9\%$) ($p < 0.05$) (Figure 2A).

DT of fibroblasts cultured in F:D 10% HS is lower compared to cells cultured in F:D 10% FBS for all passages from P0 to P3. The lowest DT was shown by the fibroblasts cultured at P1 in F:D 10% HS (1.99 ± 0.1 days), which is significantly lower than the DT of cells cultured in F:D 10% FBS (2.86 ± 0.2 days). Because fibroblasts from P1 cultured in F:D 10% HS and F:D 10% FBS showed the highest culture-expanding capability compared to the fibroblast from any other passage, they were selected for cell cycle analysis studies (Figure 2B).

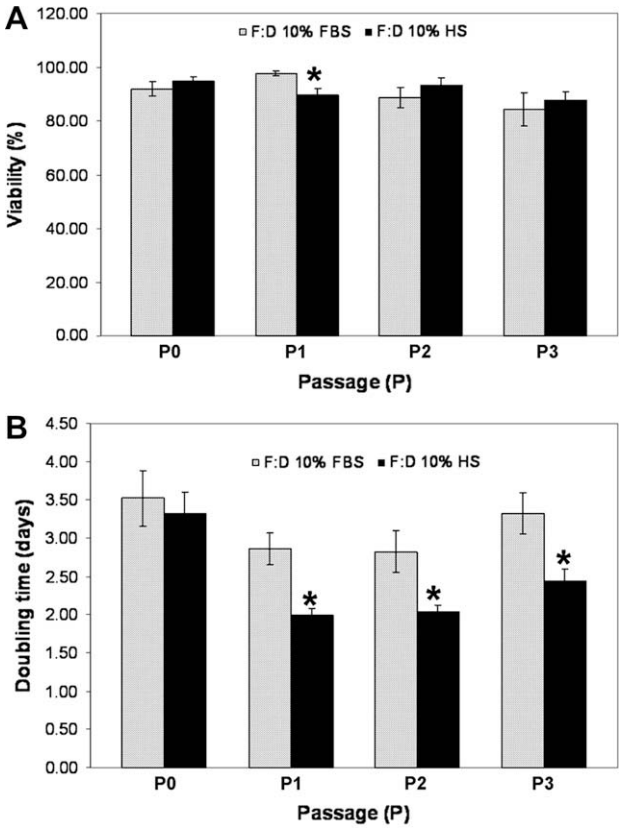

Figure 2. (A) Viability of dermal fibroblasts shows the same pattern during culture in P0, P2 and P3 where dermal fibroblasts cultured in F:D 10% HS have higher viability compared to cells cultured in F:D 10% FBS except for P1. Dermal fibroblasts cultured in 10% FBS supplementation have higher viability compared to 10% HS culture during P1 culture. $p < 0.05$ compared to F:D + 10% FBS, $n = 6$. (B) Doubling time (DT) of dermal fibroblasts generally shows similar patterns where dermal fibroblasts cultured in F:D 10% HS have lower DT compared to cells cultured in F:D 10% FBS in all passages from P0 to P3. $n = 6$. $^*p < 0.05$, P1 F:D + 10% HS compared to P1 F:D + 10% FBS. $^*p < 0.05$ P2 F:D + 10% HS compared to P2 F:D + 10% FBS. $^*p < 0.05$ P3 F:D + 10% HS compared to P3 F:D + 10% FBS.

Cell Cycle Analysis of Dermal Fibroblasts

Determining the cell cycle analysis and DNA content is very important in ascertaining that the cultured cells remain ploidy during cell culture and do not have properties of abnormal DNA that may lead to generation of cancer-cell properties. DNA histograms shown in Figure 3 were generated from fibroblasts grown *in vitro* in F:D 10% + HS medium (Figure 3A) and F:D + 10% FBS medium (Figure 3B) at P1. The percentages of cells in G0−G1, S, and G2 M (normal cell cycle step during mitosis) was determined by the ModFit software for cell cycle distribution. Fibroblasts cultured from both F:D 10% + HS and F:D 10% + FBS at P1 (12−14 days) demonstrated no evidence of aneuploidy or tetraploidy. Cells cultured in F:D 10% + HS medium demonstrated a significant increase in percentage of S-phase (synthesis phase, synthetic) ($17.12 \pm 3.8\%$) as compared to cells cultured in F:D 10% + FBS medium ($5.71 \pm 1.2\%$) (Table 2). The ratios of G0-G1 phase ($92.34 \pm 1.3\%$) for fibroblasts cultured in F:D + 10% FBS medium were significantly higher compared to fibroblasts cultured in F:D 10% + HS medium with ratios of G0-G1 phase ($82.96 \pm 3.7\%$). The ratios of G2-M phase ($1.96 \pm 0.3\%$) for fibroblasts cultured in F:D + 10% FBS medium were lower compared to fibroblasts cultured in F:D 10% + HS medium with ratios of G2-M phase ($3.49 \pm 0.3\%$). In general, DNA content percentage for fibroblasts cultured in F:D medium supplemented with 10% HS have higher S-phase ratio compared to fibroblasts cultured in F:D medium supplemented with 10% FBS, indicating higher DNA content and more cell nuclei, thus active in proliferation stage.

Quantitative Gene Expression of Human Dermal Fibroblasts

From the data observed, mRNA expression level of collagen type I was significantly lower at every passage compared to expression level from fresh digest fibroblast cells from dermis. During the monolayer culture from P0−P3, mRNA expression level of type I collagen was maintained at all passages. Expression levels were higher in fibroblasts cultured in HS compared to FBS at P0 (1.1-fold higher), and expression level of fibroblasts cultured in 10% FBS was higher compared to fibroblasts cultured in supplementation of 10% HS from P1−P3 (Figure 4A). The highest expression level obtained from cultured fibroblasts was at P2, but it was only 2.3-fold lower than fibroblasts from the fresh extract. The lowest is from fibroblasts cultured in 10% HS supplementation at P3, 4.1-fold lower than the expression levels of type I collagen from fresh digest. However, levels were not significant when compared throughout the passages.

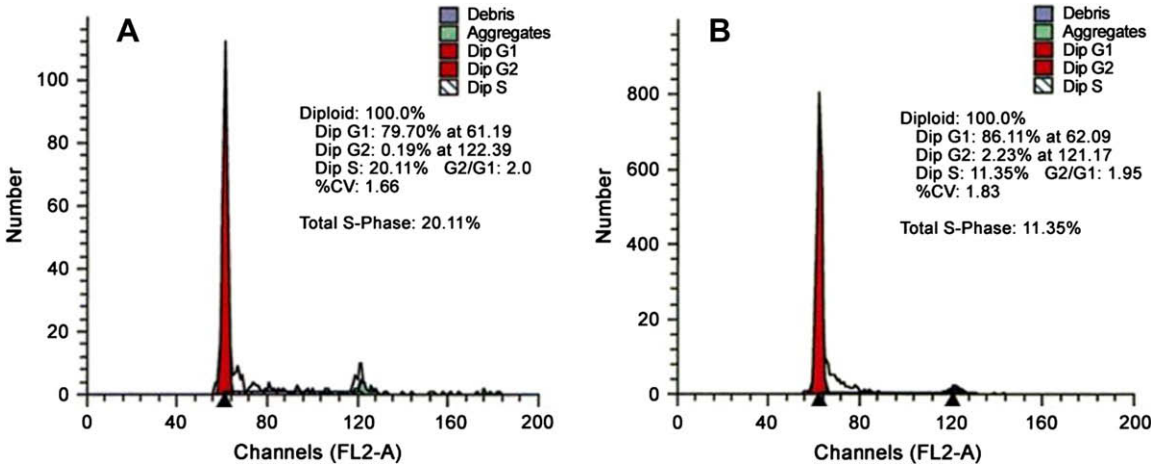

Figure 3. Example of DNA histogram generated from dermal fibroblasts grown *in vitro* showing the percentages of G0–G1, S, and G2 M phases. X-axis represents the relative fluorescence intensity proportional to DNA content. (A) DNA histogram for dermal fibroblasts cultured in F:D medium supplemented with 10% HS. (B) DNA histogram for dermal fibroblasts cultured in F:D medium supplemented with 10% FBS. Notice from the data analysis that no ratio of anueploidy or tetraploidy was detected in both histograms, indicating cells are all in diploid state (100% for both cells). DNA histogram for dermal fibroblasts cultured in F:D medium supplemented with 10% HS have higher S-phase ratio compared to dermal fibroblasts cultured in F:D medium supplemented with 10% FBS. Color version of this figure available online at www.arcmedres.com

There was no type III collagen mRNA expression detected in the freshly digested fibroblast cells from dermis (Figure 4B). However, there is an increase in the expression level from P0 to P2, while the expression level drops at P3. However, it was noticed that type III collagen mRNA expression levels for fibroblasts cultured in supplementation of 10% HS was higher at all passages (significant at P2) (4.8 ± 0.25) compared to fibroblasts cultured in supplementation of 10% FBS and was maintained throughout all passages.

From real-time PCR results, mRNA expression levels of fibronectin were increased from P1 to P3 and lower at P0 when compared to expression levels of fibroblasts from fresh digested tissue (Figure 4C). Expression levels of fibroblasts cultured in F:D 10% HS were higher from P0 to P3 (significant at P2) compared to cells cultured in supplementation of 10% FBS. The highest was achieved at P3 culture for supplementation with both 10% HS and FBS. The highest was fibroblasts cultured with 10% HS supplementation at P3, 3.5-fold higher than freshly digested fibroblasts, whereas the lowest was fibroblasts cultured in P0 with supplementation of 10% FBS, 0.6-fold lower than fibroblasts from fresh tissue.

Table 2. Mean percentage of DNA content for dermal fibroblasts cultured in F:D medium supplemented with 10% FBS and 10% HS

	G0/G1	S	G2 + M
F:D 10% FBS	92.34 ± 1.36	5.71 ± 1.18	1.96 ± 0.3
F:D 10% HS	82.96 ± 3.66*	17.12 ± 3.77**	3.49 ± 0.34

Note: Mean percentage of DNA content for dermal fibroblasts cultured in F:D medium supplemented with 10% HS has higher S-phase ratio compared to dermal fibroblasts cultured in F:D medium supplemented with 10% FBS, indicating higher DNA content, more cell nuclei active in proliferation stage.

FBS, fetal bovine serum; HS, human serum.

*G0/G1 phase significant, $p < 0.05$ compared to F:D + 10% FBS ($n = 6$).

**G0/G1 phase significant, $p < 0.05$ compared to F:D + 10% FBS ($n = 6$).

For α-SMA-2, mRNA expression levels were higher in P0 for cells cultured in F:D 10% FBS compared to freshly digested fibroblasts. For fibroblasts cultured in supplementation of 10% HS from P0 to P3, it is significantly lower compared to freshly digested fibroblasts. For fibroblasts cultured in supplementation of 10% FBS from P2–P3, it is significantly lower compared to freshly digested fibroblasts (Figure 4D). Real-time RT-PCR analysis also showed that in culture-expanded cells from P0–P3, mRNA expression levels for α-SMA-2 decreased at all passages with fibroblasts cultured in F:D 10% FBS, having much lower levels of mRNA expression compared to fibroblasts cultured in 10% HS. Fibroblasts cultured with 10% FBS supplementation at P0 have the highest α-SMA-2 gene expression, 1.2 times higher than freshly digested dermal fibroblasts. The lowest expression was observed in dermal fibroblasts cultured in 10% supplemented HS at P3, 5.7 times lower than the expression from fresh tissue fibroblasts.

Discussion

The use of HS in the monolayer culture is clinically favorable and has been previously described in chondrocyte cultures, which yields satisfying results in cell number expansion and tissue formation while maintaining the normal phenotype (21–27,34). The use of HS or HS produced in *in vitro* cultures is being studied, in view of a safer approach to clinical practice (35). The fear of prion-related diseases

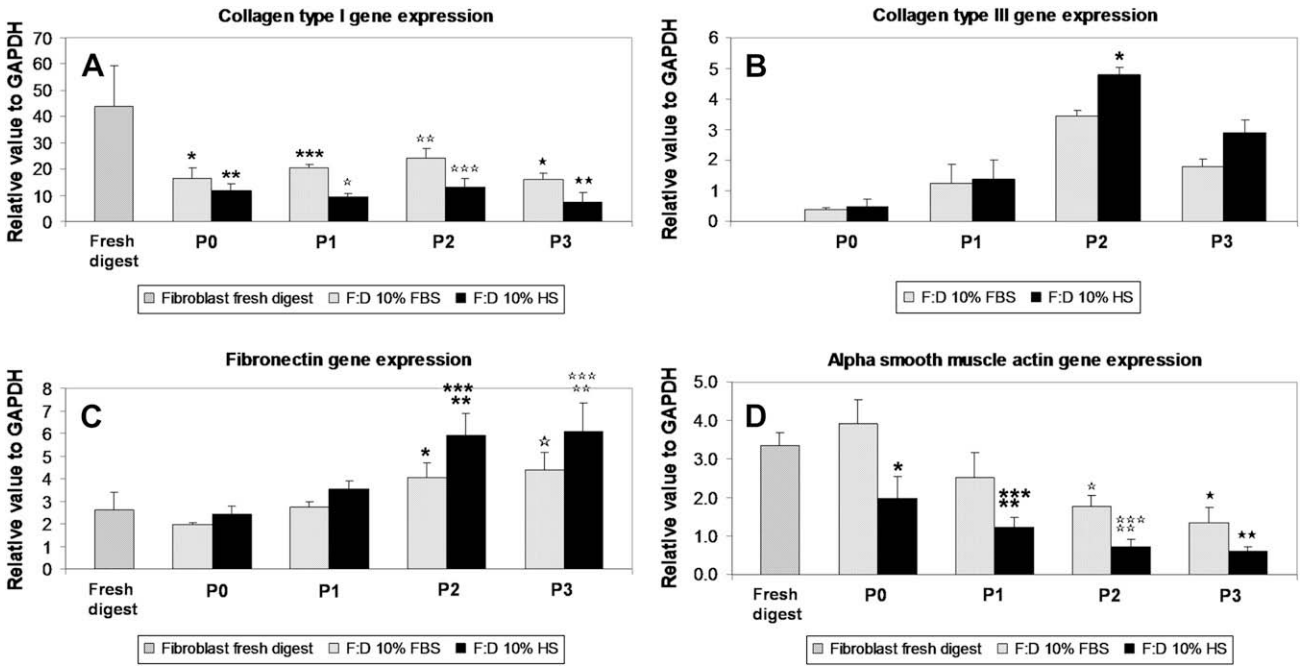

Figure 4. (A): The Collagen type I mRNA expression levels relative to GAPDH expression level of fresh digest and cultured human dermal fibroblasts in F:D 10% FBS and 10% HS from passage 0 (P0) to passage 3 (P3). Number of samples, $n=6$. *Significant, $p<0.05$, P0 F:D 10% FBS compared to fibroblasts from fresh digest. **Significant, $p<0.05$, P0 F:D 10% HS compared to fibroblasts from fresh digest. ***Significant, $p<0.05$, P1 F:D 10% FBS compared to fibroblasts from fresh digest. ☆Significant, $p<0.05$, P1 F:D 10% HS compared to fibroblasts from fresh digest. ☆☆Significant, $p<0.05$, P2 F:D 10% FBS compared to fibroblasts from fresh digest. ☆☆☆Significant, $p<0.05$, P2 F:D 10% HS compared to fibroblasts from fresh digest. ★Significant, $p<0.05$, P3 F:D 10% FBS compared to fibroblasts from fresh digest. ★★Significant, $p<0.05$, P3 F:D 10% HS compared to fibroblasts from fresh digest. (B): The Collagen type III mRNA expression levels of fresh digest and cultured human dermal fibroblasts in F:D 10% FBS and 10% HS from passage 0 (P0) to passage 3 (P3). Fresh digest was not displayed since the expression level is nil. Number of samples, $n=6$. *Significant, $p<0.05$ compared to F:D + 10% FBS. (C): The Fibronectin mRNA expression levels of fresh digest and cultured human dermal fibroblasts in F:D 10% FBS and 10% HS from passage 0 (P0) to passage 3 (P3). Number of samples, $n=6$. *Significant, $p<0.05$, P2 F:D 10% FBS compared to fibroblasts from fresh digest. **Significant, $p<0.05$, P2 F:D 10% HS compared to P2 F:D + 10% FBS. **Significant, $p<0.05$, P2 F:D 10% HS compared to fibroblasts from fresh digest. ☆Significant, $p<0.05$, P3 F:D 10% FBS compared to fibroblasts from fresh digest. ☆☆Significant, $p<0.05$, P3 F:D 10% HS compared to P3 F:D + 10% FBS. ☆☆☆Significant, $p<0.05$, P3 F:D 10% HS compared to fibroblasts from fresh digest. (D): The Alpha smooth muscle actin 2 mRNA expression levels of fresh digest and cultured human dermal fibroblasts in F:D 10% FBS and 10% HS from passage 0 (P0) to passage 3 (P3). Number of samples, $n=6$. *Significant, $p<0.05$, P0 F:D 10% FBS compared to fibroblasts from fresh digest. **Significant, $p<0.05$, P1 F:D 10% HS compared to P1 F:D + 10% FBS. ***Significant, $p<0.05$, P1 F:D 10% HS compared to fibroblasts from fresh digest. ☆Significant, $p<0.05$, P2 F:D 10% FBS compared to fibroblasts from fresh digest. ☆☆Significant, $p<0.05$, P2 F:D 10% HS compared to P2 F:D + 10% FBS. ☆☆☆Significant, $p<0.05$, P2 F:D 10% HS compared to fibroblasts from fresh digest. ★Significant, $p<0.05$, P3 F:D 10% FBS compared to fibroblasts from fresh digest. ★★Significant, $p<0.05$, P3 F:D 10% HS compared to fibroblasts from fresh digest.

in bovine products has been a problem from a regulatory standpoint (36). Although use of FBS in the production of human biological medicinal products is allowed, its use is under strict regulations. One example is the Committee for Proprietary Medical Products, which was integrated in the European Agency for the Evaluation of Medical Products (2003) (37). This Committee established the general principles that should be applied to control quality and safety of bovine serum used during the manufacturing of human biological medicinal products. This Committee also recommends the replacement of FBS with non-animal origin material or the reduction of its use. Thus, use of HS for replacement of FBS will be of major benefit in the production of human tissue-engineered products. A previous study by Coulomb et al. (38) has shown that HS contains considerable amounts of insulin-like growth factor-1 (IGF-I), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor-AB (PDGF-AB), interleukin-1α, and transforming growth factor-β1 and β2 (TGF-β1 and β2), which are important in wound healing and skin tissue reconstruction. The treatment process of HS in this study only involved sterile filtering to remove potential contamination of pathogens during HS preparation. This can minimize any loss of intrinsic growth factors needed for the growth of human dermal fibroblasts in this study.

We used HS from one healthy donor for this study because we are not allowed to withdraw autologous HS from patients who are undergoing surgery. Even though the use of autologous HS is more favorable for autologous tissue-engineered skin reconstruction, the use of a healthy donor may be an alternative to culture dermal fibroblasts, especially in the event

of major skin loss such as with burn injuries where patients suffer loss of bodily fluids. This will enable the use of screened donor HS that can be obtained from blood banks. Our results generally revealed that fibroblasts cultured in F:D medium supplemented with 10% HS have higher viability and shorter DT compared to F:D supplemented with 10% FBS, except for fibroblasts at P1. Fibroblasts cultured in supplementation of 10% FBS displayed significantly higher viability compared to fibroblasts cultured in supplementation of 10% HS. From the results, fibroblasts cultured in supplementation of 10% HS showed $<$1-fold of DT compared to fibroblasts cultured in 10% FBS, indicating higher proliferation rate. These advantages of HS over FBS in promoting fibroblast cell growth may be due to the existence of homologous growth factors in HS compared to foreign growth factors and foreign proteins in FBS. Interaction between these homologous growth factors in HS certainly increases biochemical activities in the cells, thus increasing the culture-expanding availability of the cultured fibroblasts. This will prove useful and safe especially in autologous skin substitute reconstruction where the cells must be expanded quickly in large amounts for construct formation. By multiplying the DT and total number of harvested cells at each passage, the overall fibroblasts cultured in F:D medium supplemented with 10% HS from P0–P3 has a 152.78 times culture-expanded capability in *in vitro* culture compared to 78.39 times by fibroblasts cultured in F:D supplemented with 10% FBS from P0 to P3, nearly twice within 14 days of culture.

Increasing the expansion capability of cultured human skin cells is crucial in tissue engineering. To ensure that the cultured skin cells are safe before being used for clinical applications, the cell cycle must be monitored (15,39). Because tissue-engineered products usually use rapidly growing cultured-expanded cells, it is important to maintain normal DNA content and to ensure that cultured-expanded cells do not behave as malignant or having properties of cancer cells. Cell cycle analysis using flow cytometry also can determine the percentages of the cell undergoing various phases such as G0, G2, S-phase and G2 + M (mitosis) during cell division, thus indicating whether cell is proliferating rapidly in the culture process. We used flow cytometry to monitor the cell cycle of fibroblasts cultured in both mediums supplemented with 10% HS and 10% FBS. From our results, fibroblasts cultured *in vitro* at P1 demonstrated a normal diploid state. The results demonstrated that the S-phase in the fibroblasts cultured with 10% HS supplementation is more compared to fibroblast cultured with 10% FBS supplementation. The increase in the S-phase reflected increased synthesis of DNA and increased number of cell populations in response to HS supplementation. Existence of aneuploidy or tetraploidy was not detected, thus proving the cells cultured in medium supplemented with both 10% FBS and 10% HS do not demonstrate carcinoma cells.

The results from quantitative RT-PCR studies for fibroblast monolayer culture from P0–P3 in supplementation of 10% HS compared to 10% FBS demonstrated significantly higher gene expressions of fibronectin and type III collagen and reduced α-SMA-2. Collagen type I is important as the major component providing tensile strength to skin (7). Type I collagen gene expression levels were maintained throughout the culture from P0 to P3 with higher gene expression levels at P0, although not significant in fibroblasts cultured in 10% HS compared to 10% FBS. This demonstrated that the cultured-expanded fibroblasts have the ability to maintain gene expression of type I collagen when compared to cells from native skin. In normal skin, the synthesis of type III collagen was initiated during the early phase of wound healing as part of the extracellular matrix for granulation tissue formation. Fibronectin is one of the important component for granulation tissue in addition to collagen and hyaluronic acid for attachment of cells such as macrophages and fibroblasts at the wound site in angiogenesis (40). The increased level of type III collagen and fibronectin gene expression showed that fibroblasts cultured with supplementations of HS influenced the genes of proteins involved during the wound healing process. Myofibroblasts (granulation tissue fibroblasts) are characterized by expressing high levels of α-SMA protein (41). Previous studies of myofibroblasts harvested from hypertropic scars cultured in a collagen gel lattice showed a high amount of contracture that was statistically significant compared to dermal fibroblasts cultured in a collagen gel lattice, showing that scar contracture of the hypertrophic scar is primarily provided by myofibroblasts (42,43). Increased expression of α-SMA is also regulated by growth factors in the serum, particularly by TGF-β1, which is also an important component during angiogenesis and wound healing (41,44). Both HS and FBS should contain considerable amounts of TGF-β1 that can induce the α-SMA-2 gene expressions. The lower level of α-SMA-2 gene expressions, especially in fibroblasts cultured in 10% HS, showed HS also influenced the genes involved in the formation of skin contraction and scar formation, thus suitable for use in skin substitute formation. However, our present study only concentrated on the effects of HS and FBS supplementation on the cultured fibroblast gene expression levels of wound-healing extracellular matrix protein. Further studie should be conducted at the protein level to confirm the correlations of mRNA expression of extracellular matrix protein productions from the cultured cells.

Collagens also have been used as one of the biomaterials for tissue engineering and as a coat to enhance cell attachments (1,4). The use of high collagen-producing culture-expanded human fibroblasts cells provides advantages in certain treatments such as dermal augmentation by replacing the use of bovine collagen as the main collagen-based dermal filler in the market, thus providing a safer approach in clinical applications (45,46). This will promote the use of

fully autologous dermal augmentation systems if the autologous cells are culture expanded in supplementation of patient's serum.

HS supplementation provides good culture-expanded fibroblasts that proliferate rapidly, maintaining gene expression levels of type I collagen with increased expression levels at early passage (P0) and upregulating the gene expression of type III collagen and fibronectin. This is important in downregulating gene expressions of α-SMA and extracellular matrix protein involved in wound contracture and scar formation. Use of HS for culture-expanding the fibroblasts will provide a safer approach for clinical use of fibroblasts in the formation of a skin substitute.

In conclusion, human dermal fibroblasts cultured in HS demonstrated higher expanding capability and still maintained normal cell cycle. HS supplementation influences gene expressions of extracellular matrix proteins, which are involved during the wound-healing process, such as type I collagen, type III collagen, fibronectin and α-SMA. Thus, HS is a more advantageous supplement compared to FBS. This is an important finding for the future of autologous tissue-engineered skin.

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