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Human Cord Serum as a Fetal Bovine Serum Substitute for the Culture of Human Amnion-Derived Stem Cells

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인간의 양막유래 줄기세포의 체외 배양 시 소태아혈청 대체제로서의 인간제대혈청

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ABSTRACT : Mesenchymal stem cells (MSC) are promising candidates for cell-based therapies. One major obstacle for their clinical use is the unsafety of fetal bovine serum (FBS), which is a crucial part of all media currently used for the culture of MSC. We investigated the effect of human cord serum (HCS) on the growth response, mRNA and protein expressions of human amnion-derived stem cells (HAM). HAM were isolated from the amnion after a Caesarean section and cultured in DMEM supplemented with 10% FBS, 5% HCS or 10% HCS. During culture, their biological characteristics at earlier and later passages were analyzed using RT-PCR and immunocytochemistry. Regardless of serum sources, HAM showed the prominent expression of Oct-4, Rex-1, SCF, FGF-5, BMP-4, nestin, GATA-4, NCAM and HLA ABC genes. The expression profile was observed even at later passages. Similarly, HAM cultured in either FBS or HCS exhibited the distinct protein expression of collagen I, II, III and XII, fibronectin, α-smooth muscle actin, vimentin, CK18, CD54, FSP, TRA-1-60, SSEA-3, -4 and HLA ABC. However, desmin expression was only observed in HAM cultured in medium supplemented with FBS and vWF expression was only found in HAM cultured in medium supplemented with HCS. Overall pattern of gene and protein expression of HAM was typical of known adult stem cells such as bone marrow-derived MSC. In conclusion, HCS could be as effective as FBS for the culture of HAM.

Key words : HCS, FBS, HAM, Gene expression, Cell therapy.

요 약 : 중간엽 줄기세포 (MSC)를 체외배양할 때 사용하는 소태아혈청 (FBS)의 생물학적 불안전성은 이를 임상적으로 사용하는데 있어 제한점으로 작용한다. 본 연구에서는 소태아혈청 및 인간의 제대혈청을 사용하여 인간의 양막유래 중간엽 줄기세포 (HAM)를 각각 배양한 후, 세포의 성장속도와 유전자 및 단백질의 발현 양상을 비교하였다. 제왕절개 후 얻은 양막으로부터 HAM을 분리하여 10% FBS, 5% HCS 혹은 10% HCS가 각각 첨가된 DMEM 배양액에서 배양하였으며, 초기와 후기 계대의 세포를 얻어 이들의 생물학적 특성을 비교 분석하였다. 역전자 중합효소반응 결과, 혈청의 종류에 상관없이 배양된 세포들은 모두 OCT-4, Rex-1, SCF, FGF-5, BMP-4, nestin, NCAM, GATA-4, HLA-ABC 유전자를 발현하였으며, 이러한 발현은 초기 및 후기 계대의 세포에서도 마찬가지로 나타났다. 세포면역화학 반응 결과, FBS 혹은 HCS를 첨가한 배양액에서 배양된 HAM은 4번째 계대에서 collagen I, II, III, XII, fibronectin, α-smooth muscle actin, vimentin, CK18, CD54, FSP, TRA-1-60, SSEA-3, -4, HLA ABC 단백질을 뚜렷하게 발현하였다. 그러나 desmin 단백질은 FBS가 첨가된 배양액에서 배양된 HAM에서만 발현되었고 vWF 단백질은 HCS가 첨가된 배양액에서 배양된 HAM에서만 발현되었다. 결론적으로 유전자와 단백질의 발현양상을 살펴본 결과, HCS가 첨가된 배양액에서 배양된 HAM은 전형적인 인간성체줄기세포의 특징을 나타내고 있으며, FBS가 첨가된 배양액과 비교하여 동등한 성장 촉진 효과를 가지는 것으로 보인다.

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INTRODUCTION

Adult stem cells are thought to be present in all tissues and are responsible for maintaining the specific tissue in

which they reside. In adults, the most typical organ bearing stem cells is bone marrow, which contains two stem cell populations: haematopoietic stem cells (HSC) and mesenchymal stem cells (MSC). HSC are capable of regenerating all haematopoietic cell lineages and have been used in clinical medicine since 1970's in bone marrow transplantation after cytotoxic therapies. A number of studies in recent years have demonstrated that their differentiating capabilities often extend to other cell types as well. However, their full differentiation potential has yet to be elucidated, and their sources are restricted to bone marrow, umbilical cord blood, and peripheral blood. MSC isolated from bone marrow (BM-MSC) can differentiate into cell lines of different origin. They can become (a) mesodermal cell lines, such as osteocytes, chondrocytes, adipocytes (Pittenger *et al*., 1999), and skeletal myoblasts, (b) endothelial cell lines (Reyes *et al*., 2001), (c) ectodermal cell lines, such as neurons (Woodbury *et al*., 2000), and (d) endodermal cell lines, such as hepatocyte-like cells (Schwartz *et al*., 2003). However, multipotent stem cells, with differentiation potential similar to BM-MSC, have been found in connective tissue (Young *et al*., 2001), adipose tissue (Gronthos *et al*., 2001), muscle (Jankowski *et al*., 2002), synovial membranes (De Bari *et al*., 2001), vascular elements in deciduous teeth (Miura *et al*., 2003), peripheral blood (Kuznetsov *et al*., 2001), and skin (Shih *et al*., 2005). They are also found in tissues that are normally discarded at the time of birth, such as umbilical cord (Mitchell *et al*., 2003; Romanov *et al*., 2003), cord blood (Lee *et al*., 2004), placenta (Yen *et al*., 2005), amniotic fluid (In't Anker *et al*., 2003), and amniotic membrane.

The amniotic membrane consists of a single layer of amniotic epithelial cells that reside on a basement membrane, with an underlying layer containing stromal cells, principally mesenchymal cells (HAM). HAM, which are also derived from the epiblast of the inner cell mass have been shown to express the Oct-4 gene, a specific marker of embryonic stem cells (Zhao *et al*., 2005), implying the presence of a primitive form of stem cell. Indeed, they can differentiate into neuroglial-like cells *in vitro* (Sakuragawa *et al*., 2004), cardiomyocyte-like cells both *in vitro* and *in vivo* (Zhao *et al*., 2005), osteoblasts, adipocytes and chondroblasts (Portmann-Lanz *et al*., 2006). These observations collectively demonstrate that HAM could act as primitive MSC capable of differentiating into multilineage cells, including mesodermal and ectodermal tissues.

MSC can currently expand *in vitro*, either under experimental or clinically grade conditions, in the presence of 10～20% fetal bovine serum (FBS), which is considered crucial for the expansion (Catersion *et al*., 2002; Sotiropoulou *et al*., 2006). FBS characteristics are routinely pre-screened to guarantee both the optimal growth of MSC and the bio-safety of the cellular product. Despite these, the use of FBS raises some concerns particularly when utilized in clinical grade cellular preparations, since the administration of animal products to humans might theoretically cause the transmission of prions and still unidentified zoonoses. Moreover, bovine proteins or peptides might be incorporated by MSC during culture procedures (Spees *et al*., 2004; Gregory *et al*., 2006) and provoke immune reactions in the host, especially when repeated infusions are needed, with consequent rejection of the transplanted cells (Horwitz *et al*., 2002). As a result, several countries have legislated warnings and restrictions on the clinical use of cell therapy products prepared in the presence of FBS.

Human cord serum (HCS) is a rich source of different cytokines required for growth and survival of different types of stem cells like HSC. Lam *et al*. (2001) have used autologous cord blood plasma for the expansion of cord blood HSC. Autologous cord blood plasma has also been used to culture T cells for adoptive immunotherapy of growth factors such as insulin-like growth factor (IGF), transforming growth factor-β (TGF-β) and vascular endothelial growth factors (VEGF).

Throughout this study, we investigated the suitability of pooled HCS as a replacement for FBS to develop clinically useful protocols for MSC expansion. We also investigated

the optimal conditions for *ex vivo* expansion of MSC using various growth factors.

MATERIALS AND METHODS

1. Chemicals

All antibodies used in this study were mouse monoclonal antibodies. Anti-human HLA ABC and HLA DR antibodies were purchased from Novo Castra (Newcastle, UK). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless noted otherwise.

2. Preparation of Human Cord Serum (HCS)

The human cord serum that would normally be discarded was collected after delivery from pre-screened mothers. These women were screened for infectious disease, including HIV 1 and 2, hepatitis B and C viruses, and other sexually transmitted disease. The human cord serum was stored only with the mother's signed consent as per the institutional review board, and no collection was made if there were any complications during delivery.

The human cord blood was allowed to clot at 4℃ for 12～24 h. The blood was then centrifuged at 3,000 rpm for 30 min at 4℃ and the clear serum was collected into sterile containers. The hemolysed serum did not use in this research and the cleared serum was stored at －70℃. Complement was inactivated by keeping the serum at 56℃ for 30 min. Serum from a minimum of three donors was pooled to eliminated batch variations due to donor characteristics. Serum was filter sterilized by passing through a 0.22 μm filter (Millipore, Bedford, MA).

3. Isolation of HAM

Placentae were harvested within 4 h of delivery after cesarean section from the volunteers who had given informed consent. The fresh amnion was mechanically separated from the chorion and washed three times in phosphate-buffered saline (PBS, Gibco BRL, Grand Island, NY) to remove blood and cellular debris. The amnion was cut into 5×5 cm^2 pieces. After mincing, they were placed in a spinner flask, containing low-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 0.25% trypsin (Gibco). The flasks were incubated at 37°C with stirring for 30 min. The supernatant from the first trypsin digestion, which consists primarily of red blood cells, was discarded after centrifugation. The residual tissue was minced and incubated again at 37℃ with 0.25% trypsin for 30 min with stirring. Mostly epithelial cells were liberated in this round of trypsinization, and these were removed by centrifugation and the procedure was repeated once again. Using these methods, more than 98% of the epithelial cells, as determined microscopically, were removed. HAM cells were isolated from the remaining amnion tissue pieces. The tissue pieces were placed in PBS, containing 2 mg/mL collagenase A (Roche Diagnostics, Rotkreuz, Switzlerland) and 0.05 mg/mL DNase (Roche) and incubated at 37℃ for 2 h with stirring. The dispersed mesenchymal cells were collected by centrifugation at 500 g. After several washes with DMEM, the cells were plated in 75 mL culture flasks (Nunc, Rochester, MN), containing DMEM supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 3.7 mg/mL sodium bicarbonate, and 10% FBS (Gibco). The culture medium was changed once a week until fibroblast-like cells appeared and twice a week thereafter. For the subculture, cells achieving confluence up to 3 weeks were treated with 0.125% trypsin and 1 mM ethylenediamine tetraacetic acid (EDTA) for 3 min, and the released cells were collected and cultured again until they reached senescence. Morphologically homogeneous populations of fibroblast-like cells, i.e. HAM, were obtained after 1～2 subcultures. Some of cells at each passage were pelleted and stored at －20℃ until use.

4. Culture of HAM and Treatment of Growth Factors

HAM were culture in 10% FBS, 5% HCS or 10% HCS during *ex vivo* expansion. HAM also were cultured in 10% HCS supplemented with 10 ng/mL epidermal growth factor (EGF; Peprotech, Princeton, NJ), 5 ng/mL fibroblast

growth factor-2 (FGF-2; Peprotech) or 10 ng/mL insulin-like growth factor-1 (IGF-1; Peprotech).

5. Immunocytochemistry

HAM were plated on Lab-Tek chamber slides (Nunc), and fixed with 4% paraformaldehyde in PBS at 4℃ for 2 h. Cells were permeabilized with PBS, containing 0.5% Triton X-100 for 10 min at room temperature. After several washings, the slides were incubated in 3% hydrogen peroxidase for 15 min to quench the endogenous peroxidase activity, followed by rinsing with PBS and incubating in a blocking solution (2% BSA in PBS) for 1 h at room temperature. The slides were then incubated with primary antibodies mouse anti-HLA ABC, 1:100, and mouse anti-HLA DR, 1:50 17 h at 4℃, followed by three rinses with PBS and incubation with biotinylated goat anti-mouse or rabbit immunoglobulin G (IgG) (Dako, Carpinteria, CA) for 20 min at room temperature. After rinsing with PBS to remove the secondary antibody, the slides were then incubated with horseradish peroxidase conjugated streptavidin (Dako) for 20 min at room temperature. Immunoreactivity of each protein was visualized, using 3,3'-diaminobenzidine tetrahydrochloride (Dako) and counterstaining with Mayer's haematoxylin. The final slides were photographed under a microscope, using bright-field illumination (LSM410, Carl Zeiss, Oberkochen, Germany).

6. Total RNA Isolation and Reverse Transcription -Polymerase Chain Reaction (RT-PCR)

All solutions were prepared using distilled water treated with 0.1% diethylpyrocarbonate. HAM cell pellets were washed with Ca^{2+}/Mg^{2+}-free PBS and transferred to a chilled Eppendorf tube on ice. Five hundreds microliters of Tri-reagent was immediately added to the tube, and the tubes were stored at −20℃ until use. Total RNA was isolated according to the manufacturer's instructions (Sigma). The RNA was allowed to stand at 65℃ for 5 min in a heating block before being chilled on ice and quantified spectrophotometrically. The purity of the RNA was assessed by determining the ratio of absorbance at 260 nm to that at 280 nm (>1.8). RT-PCR was performed using a GeneAmp PCR system 2400 (Perkin Elmer).

Five micrograms of total RNA per sample was subjected to reverse transcriptase in a 20 μL sample volume, consisting of 1 x reaction buffer, 1 mM dNTPs mixture, 0.5 μg/μL oligo $(d)T^{15}$, 20 U RNase inhibitor (Takara, Japan), and 20 U M-MuLV reverse transcriptase (Fermentas, Burlington, Canada). The RT reaction occurred at 42℃ for 60 min, and the resultant cDNA subjected to PCR amplification using gene-specific upstream and downstream primers (Table 1). PCR was performed in a 10 μL sample volume, consisting of 2 mM $MgCl_2$, 1×*Taq* buffer, 0.25 U *Taq* polymerase (Takara), and 10 pM each of the upstream and downstream primers. Amplification was performed for 35 cycles at a denaturing temperature of 94℃ for 30 sec and an extension temperature of 72℃ or 68℃ for 30 sec. The annealing temperature was determined by the primer sequence. The PCR products were mixed with 6×loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose) and resolved on a 2% agarose gel. After the gel was stained with ethidium bromide, DNA signals on the gel were visualized under ultraviolet light using a Bio-profile image analysis system.

RESULTS

1. HAM Cultured in HCS were Phenotypically Similar to Those Cultured in FBS

Primary cultures of cells isolated from the amnion were heterogeneous but consisted primarily of two types of cells: one similar to fibroblast and the other similar to epithelial cell. Fibroblast-like cells sometimes appeared as colonies in the culture flask during the primary culture. After 10～12 days of culture, the culture flask was confluent with a heterogeneous population which, when subcultured, yielded mostly fibroblast-like cells that closely resembled the BM-MSC. These fibroblast-like cells, named as HAM, persisted their morphology throughout the culture

Table 1. cDNA pairs used in the present study

Gene	Primer sequence	Accession number	Size(bp)	Annealing temperature
GAPDH	5'- aca act ttg gta tcg tgg aa-3' 5'- aaa ttc gtt gtc ata cca gg-3'	NM_002046	456	55
Oct-4	5'- cgt gaa gct gga gaa gga gaa gct g -3' 5'- caa ggg ccg cag ctc aca cat gtt c -3'	AF268617	245	55
Rex-1	5'- atg gct atg tgt gct atg agc -3' 5'- cct caa ctt cta gtg cat cc -3'	NM_174900	449	57
SCF	5'- cca ttg atg cct tca agg ac -3' 5'- ctt cca gta taa ggc tcc aa -3'	M59964	275	55
FGF-5	5'-gct gtg tct cag ggg att gta gga ata -3' 5'-tat cca aag cga aac ttg agt ctg ta -3'	NM_004464	434	55
Nestin	5'-cca gaa act caa gca cca c -3' 5'-ttt tcc act cca gcc atc c -3'	X65964	398	54
BMP-4	5'-agc cat gct agt ttg ata cc -3' 5'-tca ggg atg ctg ctg agg tt -3'	D30751	383	55
Brachyury	5'-gag ctc acc aat gag atg at -3' 5'-ggc tca tac tta tgc aag ga -3'	NM_002052	335	57
GATA-4	5'-ttc ctc ttc cct cct caa at -3' 5'-tca gcg tgt aaa ggc atc tg -3'	NM_002052	194	60
BMP-2	5'-ttg cgg ctg ctc agc atg tt -3' 5'-ttg cga gaa cag atg caa gat g -3'	BC069214	315	55
NCAM	5'-gag ggg gaa gat gcc gtg atg tg -3' 5'-ata ttc tgc ctg gcc cgg atg g -3'	NM181315	269	60
HLA ABC	5'- gta ttt ctt cac atc cgt gtc ccg -3' 5'- gtc cgc cgc ggt cca aga gcg cag -3'	L18898	394	70
HLA DR	5'- ctg atg agc gct cag gaa tca tgg -3' 5'- gac tta ctt cag ttt gtg gtg agg gaa g -3'	X06079	220	60

period before they reached senescence (Fig. 1C, 1F, 1I). Cells isolated and expanded in 5% (Fig. 1D, 1E, 1F) or 10% HCS (Fig. 1G, 1H, 1I) were morphologically indistinguishable from those cultured in 10% FBS (Fig. 1A, 1B, 1C). Particularly, morphological differences were not discernible between cells expanded 10% HCS and 10% FBS in size, fibroblast-like appearance and colony-formation. HAM cultured in 5% HCS were shorter than cells in 10% HCS. It appeared that HAM grown in 10% HCS detached more readily from plastic culture flask than cells in FBS when they were exposed to trypsin and EDTA. However, when cell adhesion molecules such as CD44, CD54 and CD106 were determined by immunocytochemistry, the proteins were almost equally expressed in FBS or HCS as shown in Table 2.

2. HAM Efficiently Expanded in the Presence of HCS

To examine the effect of HCS on proliferative capability of HAM, cells from primary culture were set in parallel cultures supplemented with one of three preparations of 10% FBS, 5% HCS or 10% HCS. When the culture was expanded, HAM in 10% FBS and 10 % HCS ceased proliferation after 13 passages. The other HAM in 5% HCS stopped after 8 passages. As calculated from the number of passages and period in culture (Fig. 2A, 2B), the average doubling time of HAM from the second passage until senescence was 2.9 days for HAM in 10% FBS, 3.0 days for HAM in 5% HCS and 2.8 days for HAM in 10% HCS. HAM underwent 37.6 doublings in 10% FBS, 19.3 doublings in 5% HCS and 36.4 doublings in 10% HCS. The total accumulated number of cells was estimated to be

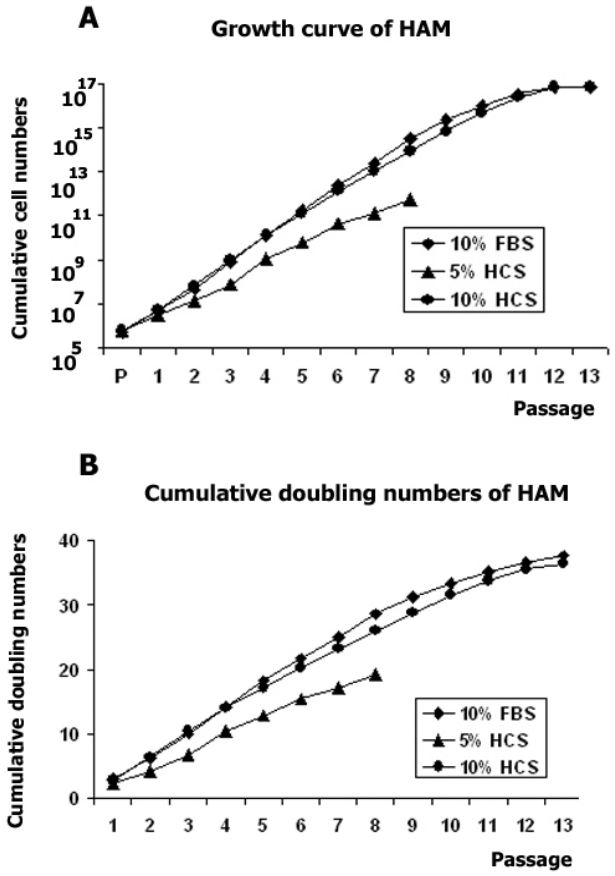

Fig. 1. Morphology of HAM cultured in medium containing FBS or HCS during *ex vivo* expansion. A, B and C; HAM cultured in 10% FBS at 2nd (A), 5th (B) or 10th (C) passage. D, E and F; HAM cultured in 5% HCS at 2nd (D), 5th (E) or 8th (F) passage. G, H and I; HAM cultured in 10% HCS at 2nd (G), 5th (H) or 10th (I) passage. Magnification, ×40.

7.0×10^{16}, 5.8×10^{11} or 6.8×10^{16}, respectively.

3. Gene Expression by HAM Cultured in HCS

Regardless of the culture condition, HAM exhibited three different patterns of mRNA expression during *ex vivo* expansion (Fig. 3). The first group of genes was expressed consistently throughout the culture period examined. These were Oct-4, Rex-1, SCF, FGF-5, nestin, BMP-4, GATA-4, and HLA ABC, and their expressions were detected by HAM in 10% FBS during 3～10 passage, HAM in 5%

Table 2. Immunocytochemical analysis of proteins expressed by HAM at 4th passage

Proteins		10% FBS	5% HCS	10% HCS
Embryonic stem cell markers	TRA-1-60	+	+	++
	SSEA-3	+	+	+
	SSEA-4	+	+	++
Mesenchymal stem cell marker	Thy-1	+	++	+
Extracellular matrix molecules	Collagen I	++	++	++
	Collagen II	+	+	+
	Collagen III	+	++	++
	Collagen IV	+	++	++
	Collagen XII	+	++	++
	Fibronectin	+	++	++
Cytoskeletal markers	αSMA	++	++	++
	Vimentin	+	+	+
	Desmin	+	-	-
	CK18	+	++	++
Surface antigens	CD44	+	++	++
	CD54	+	+	+
	CD106	-	-	-
	FSP	++	+	++
Endothelial cell markers	vWF	-	+	+
	CD31	-	-	-
HLA	HLA ABC	++	++	++
	HLA DR	-	-	-

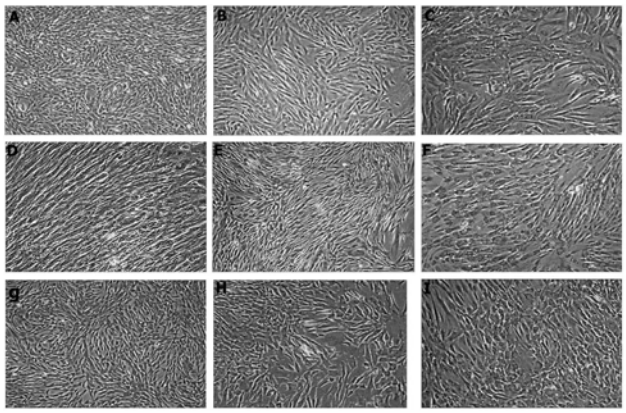

Fig. 2. Growth curves and cumulative doubling numbers of the HAM cultured in medium containing FBS or HCS. (A) Growth curve of the HAM throughout *ex vivo* expansion. (B) Cumulative doubling number of the HAM during the same culture period.

HCS during 2～6 passage, HAM in 10% HCS during 2～10 passage. The second group of genes including Brachyury and BMP-2 was never expressed by HAM in any passages. The third group of genes showed a differential expression pattern during *ex vivo* expansion. HLA DR genes were expressed by HAM cultured in 10% FBS or 10% HCS at passage 5, but not expressed by HAM cultured in 10% FBS or 10% HCS at passage 10 (Fig. 3).

4. Protein Expression by HAM Cultured in Medium Supplemented with HCS

Whether HAM were cultured in medium supplemented with HCS or FBS, immunocytochemical staining demonstrated that both kinds of cells examined at the 4th passage showed intense staining with the antibodies against the collagen I, α-SMA, FSP and HLA ABC in 10% FBS, TRA-1-60, SSEA-4, collagen Ⅰ, collagen Ⅲ, -Ⅳ, -Ⅻ, fibronectin, α SMA, CK18, CD44, CD106 and HLA ABC proteins. However, desmin protein was detected in HAM cultured only in 10% FBS. In contrast, vWF protein was detected in HAM cultured in medium supplemented with 5% or 10% HCS but not FBS.

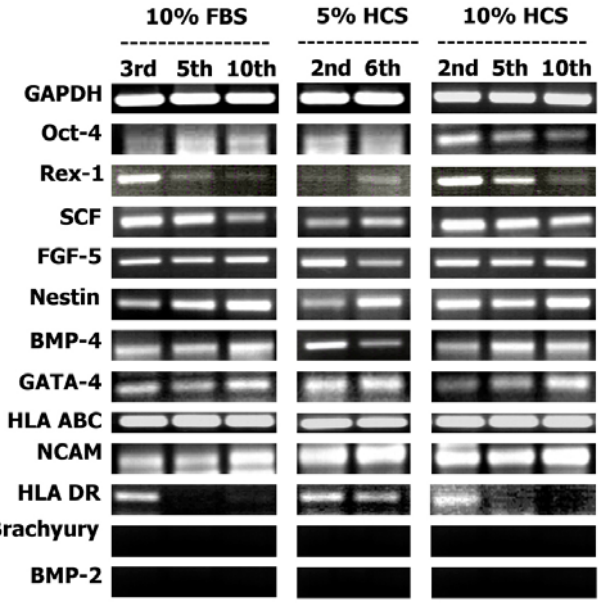

Fig. 3. Expression profiles of stem cell markers by HAM cultured in medium containing FBS or HCS. HAM were cultured in either 10% FBS, 5% HCS or 10% HCS and RT-PCR analyses were done at designated passages.

5. *Ex vivo* Expansion of HAM Cultured in HCS Supplemented with Growth Factors

To examine the effect of growth factors on proliferative capability of HAM during culture in the presence of either FBS or HCS, cells from primary culture were set into parallel cultures supplemented with one of five different preparations consisting of 10% FBS or 10% HCS supplemented with 10 ng/mL EGF, 5 ng/mL FGF-2 or 10 ng/mL IGF-1. When the culture was expanded, HAM ceased proliferation at 12.7 passages in FBS, at 12.3 passages in HCS, at 13.0 passages in HCS supplemented with FGF-2 and at 11.7 passages in HCS supplemented with EGF or IGF-1 (Fig. 4A). HAM underwent 35.8 doublings in FBS, 34.8 doublings in HCS, 31.9 doublings in HCS supplemented with EGF, 38.1 doublings in HCS supplemented with FGF-2 and 33.1 doublings in HCS supplemented with IGF-1. The total accumulated number of cells was estimated to be 8×10^{16} in FBS, 2.2×10^{16} in HCS, 2.2×10^{15} HCS supplemented with EGF, 1.3×10^{16} HCS supplemented with FGF-2 and 1.9×10^{16} HCS supplemented with IGF-1 (Fig. 4B).

From the primary culture until senescence, HAM maintained a fibroblast-like morphology. There was no discernible difference between cells expanded in FBS and HCS even in the presence of various growth factors at any time (Fig. 5).

DISCUSSION

Many insights in the MSC biology, as well as of their immune regulatory properties and regenerative potential, have been obtained in the last few years and these have provided the support for considering MSC as an attractive and powerful tool for cell therapy-based approaches (Horwitz *et al*., 2002; Le Blanc Ringden, 2005; Pountos *et al*., 2006). FBS is widely utilized to supplement culture medium in protocols designed to generate and expand MSC *in vitro*

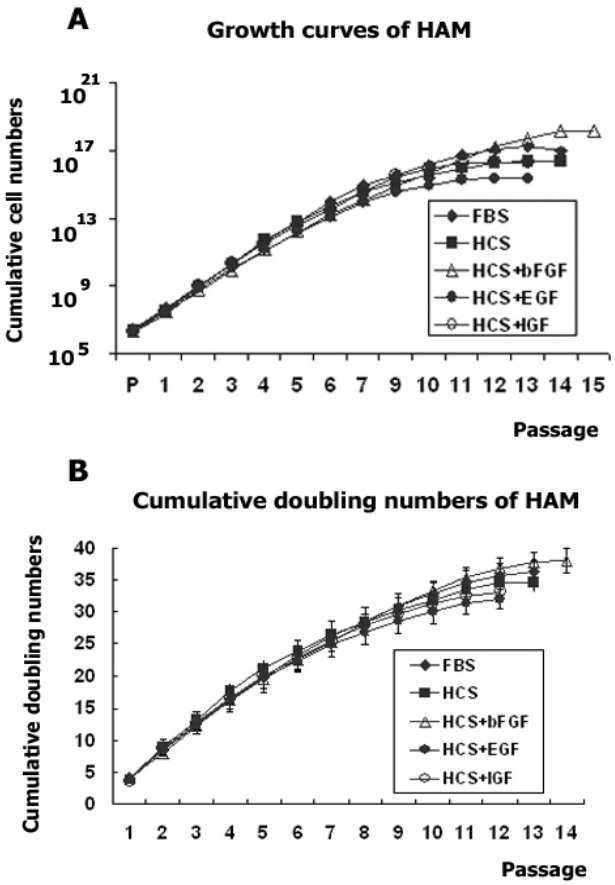

Fig. 4. Growth curve and cumulative doubling numbers of the HAM cultured in media containing FBS or HCS during *ex vivo* expansion. (A) Growth curve of the HAM throughout *ex vivo* expansion. (B) Cumulative doubling numbers of the HAM during the same culture period. HAM were cultured in 10% FBS or 10% HCS supplemented with EGF, FGF-2 or IGF-1. Results from three independent experiments are represented as the mean±s.e.m.

for the clinical use (Sotiropoulou *et al*., 2006). However, FBS is of animal origin and thus is unsuitable for infusion into human being. Therefore the development of the method for removal of exposed FBS is critical for clinical utilizations of MSC in cell-based therapies.

Increasing number of laboratory protocols using MSC underscores the need for serum supplementation other than FBS. Serum-free media have been investigated, but none has been published so far that supports the proliferation of

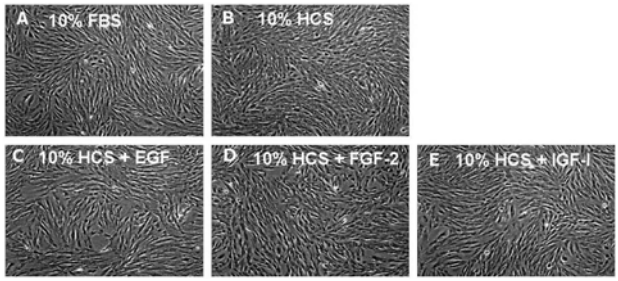

Fig. 5. Morphology of HAM cultured in medium containing FBS or HCS during *ex vivo* expansion. HAM were cultured in 10% FBS (A) or 10% HCS (B) supplemented with EGF (C), FGF-2 (D) or IGF-1 (E). All photographs were taken at 2nd passage. Magnification, ×40.

MSC in the absence of growth factors. Recently, human serum and platelet lysates have been identified as promising substitutes (Mizuno *et al*., 2006; Doucet *et al*., 2005; Stute *et al*., 2004; Yamaguchi *et al*., 2002). Platelet lysate have already been demonstrated to be a powerful source of growth factors, useful in the treatment of a variety of soft and hard-tissue surgical conditions and in the management of non-healing wounds (Marx, 2004). The utilization of platelet lysate as a culture supplement for MSC expansion in cell therapy-based protocols has been recently suggested as a promising alternative to FCS (Doucet *et al*., 2005; Kocaoemer *et al*., 2007). However, obtaining large volumes of human serum is difficult; hence it is not widely used for culturing cells, tissues and organs *in vitro*.

In this study, we have described a novel procedure to generate MSC from human amniotic membrane and to expand *ex vivo* using HSC instead of FBS. Primary cells from amnion cultured in HCS gave rise to MSC with a similar proliferation rate compared to cells cultured in conventional culture condition consisting of FBS. RT-PCR analyses of these cells showed distinct expression of the stem/progenitor cell markers such as Oct-4, Rex-1, SCF, FGF-5, nestin, BMP-4 and GATA-4, which were similarly expressed in cells cultured in FBS.

FBS-free culture of MSC will be a safer way in human MSC transplantation into patients. There has been much controversy about what constitutes a suitable FBS supple-

ment. Most studies have concluded that serum-free media cannot promote MSC growth without the addition of cytokines (Kuznetsov *et al*., 2000). The simple replacement of FBS by human serum in α-MEM media did not support proliferation of MSC (Kuznetsov *et al*., 2000), although others reported short-term growth and increased spontaneous osteogenic differentiation of MSC (Spees *et al*., 2004; Schecroun & Delloye, 2004). The persistence of xenogenic proteins in human MSC expanded in FBS was examined extensively (Spees *et al*., 2004). After intravenous administration of autologous rat MSC expanded in FBS, humoral immune responses against FBS proteins were observed in the recipient. To reduce the immunogenicity of FBS carried within human MSC, recently, several studies have concentrated on the use of autologous serum (AS) supplemented with growth factors after an initial period of expansion (Spees *et al*., 2004). Another study has described the comparison between human MSC in FBS versus AS, but only in short-term cultures and with limited phenotypic characterization (Stute *et al*., 2004). Human MSC cultured in AS can be used without any safety issues raised by FBS contamination in cellular and genetic therapies including promotion of engraftment after allogeneic HSC transplantation (Kim *et al*., 2005). Shahdadfar *et al*. (2005) showed that expansion of human MSC in AS without FBS and any cytokines is as effective as supplementing the culture medium with FBS. Yet, this approach is limited by the amount of AS necessary to expand MSC for clinical use (Sotiropoulou *et al*., 2006) and the variability of serum, particularly for patients receiving prior to chemotherapy. Mizuno *et al*. (2006) showed that AS was significantly better than FBS in expanding of marrow derived MSC. However their cells were cultured for 9 days only and therefore they have probably looked at cells in first passage only. In this study, we successfully cultivated MSC in HCS from different sources for 13～14 passages yielding little difference compared to cells cultured in FBS. Another drawback of using AS is that it may be difficult to obtain enough serum from a single donor for large scale culture of MSC. Recently human MSC showed a greater proliferation potential in the presence of HCS than in FBS (Shetty *et al*., 2007). With cord blood serum this will not be a problem since sera from multiple donors can be pooled to generate enough quantities of serum for large scale cultures. We also used pooled sera from a minimum of three donors to eliminate donor specificities of cord blood serum. Some studies have been successful in isolating and expanding MSC using adult blood serum (Anselme *et al*., 2002; Yamaguchi *et al*., 2002), however, others have reported growth arrest of MSC after first passages (Spees *et al*., 2004; Shahdadfar *et al*., 2005).

FGF-2 is a mitogen for a variety of cells including mesenchymal or neuroectodermal orgin *in vitro* (Deasy *et al*., 2002). It has been demonstrated that FGF-2 supplementation to BM-MSC cultures increases the frequency of tripotential progenitors, capable of osteogenic, chondrogenic, and adipogenic differentiation at a clonal level (Muraglia *et al*., 2000). The supplementation to BM-MSC primary cultures was effective to select a subpopulation of earlier progenitors with significantly longer life span and to maintain their differentiation potential for more than 50 population doublings *in vitro* (Bianchi *et al*., 2003). Similarly, we found that FGF-2 supplementation increased the proliferation and survival rate of HAM during cultured in HCS. These results demonstrate that FGF-2 could be also effective for the selection and the survival of a particular subset of cells enriched in pluripotent mesenchymal precursors in the presence of HCS.

In view of this study, it is suggested that HCS could replace FBS in the generation and expansion of MSC in certain cell therapy protocols. Further studies are needed whether MSC expanded in the presence of HCS might retain their multipotency to differentiate into mesodermal cell lineages, chondrocytes, osteocytes and adipocytes.

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