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Hepatic Differentiation of Human Eyelid Adipose-Derived Stem Cells

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사람의 눈지방유래 줄기세포의 간세포 분화

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ABSTRACT : A variety of stem cells has been emerging as therapeutic cells that can replace organ transplantation in human liver diseases. The present study focused on whether human eyelid adipose-derived stem cells (HAD) might differentiate into functional hepatocyte-like cells *in vitro*. HAD were isolated from human eyelid adipose tissue. Effect of dimethyl sulfoxide (DMSO), fibroblast growth factor (FGF)-2 and FGF-4 on the hepatic differentiation of HAD have been examined *in vitro*. Immunocytochemical analysis and PAS staining showed that HAD cultured in both DMSO and FGF-4 exhibited the most intense staining than HAD of the other experimental groups. These HAD expressed numerous hepatocyte-related genes. Immunoblotting analyses showed that HAD cultured in the presence of DMSO and FGF-4 secreted higher amount of human albumin than HAD cultured in other conditions. Urea analysis also demonstrated that these HAD produced higher amount of urea than any other groups of HAD. In conclusion, combined treatment of DMSO and FGF-4 could effectively induce the functional differentiation of HAD into hepatocyte-like cells.

Key words : Human adipose-derived stem cells, Hepatocyte-like cells, Albumin secretion, Urea assay, Western blotting.

INTRODUCTION

The liver, one of the largest organs in the human body, plays a central role in metabolism, detoxification, and maintenance of homeostasis in the body (Columbano et al., 1996). Proper liver functions are fundamental to human health and loss of them can have serious diseases. Among the therapies, orthotopic liver transplantation has been successfully used to treat a variety of end-stage liver diseases but limited by the lack of availability of donor organs (Miro et al., 2006). Hepatocyte transplantation is a potential way to replace organ transplantation, and to decrease mortality in acute liver failure and treated metabolic liver disease (Nussler et al., 2006). However, hepatocytes are only available from a restricted number of donor organs, and primary hepatocytes are not only difficult to replicate the sufficient number for transplantation but also to maintain their properties *in vitro* (Jones et al., 2002).

In recent years, advances in stem cell biology have made the prospect of tissue regeneration a potential clinical reality, and several studies have shown the great promise that stem cells hold for therapy (Assmus et al., 2002; Wollert et al., 2004). Mesenchymal stem cells (MSC) found in many adult tissues are an attractive stem cell source for the regeneration of damaged tissues in clinical applications because they are characterized as undifferentiated cells, able to self-renew with a high proliferative capacity, and possess a mesodermal differentiation potential (Pittenger et al., 1999). Although bone marrow (BM) has been the main source of MSC, the harvest of BM accompanied a highly invasive procedure and the number, differentiation potential and life span of MSCs declined with increasing of age (Stenderup et al., 2003). Therefore, recruitment of

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alternative sources of MSC is subject to intensive investigation.

Adipose tissue is an attractive source of MSC. It is, like BM, derived from the embryonic mesenchyme. It has been identified that the adipose stromal compartment contained a putative stem cell population and it is termed as adipose tissue-derived stem cells (ADSC) (Strem et al., 2005). These cells can be easily isolated, expanded extensively and induced to various mesenchymal tissues, including chondrocytes adipocytes, osteoblasts, myocytes, and endothelial cells (Dicker et al., 2005; Im et al., 2005). Recent reports have demonstrated that ADSC can generate hepatocyte-like cells (Seo et al., 2005; Talens-Visconti et al., 2006; Banas et al., 2007). Therefore, it will be sufficiently supply for liver disease if functional hepatocytes from stem cells were differentiated. Stem cells holds promise as a potential source of hepatocytes for cell based therapies to treat the failed liver. Many studies have shown that embryonic stem cells (ESC) (Hamazaki et al., 2001), BM derived-MSC (Schwartz et al., 2002), liver stem cells/oval cells (Gupta et al., 1992), cord blood cells (Newsome et al., 2003), and fetal hepatocytes (Malhi et al., 2002) are possible cell types that can develop into viable hepatocytes. However, ESC utilization has many limitations, such as teratoma formation followed by transplantation, immunogenicity, and ethical issues, they are restricting their clinical use (Reubinoff et al. 2000). Previously, many reports demonstrated expression of various liver-specific genes and proteins including albumin following *in vitro* culture of various adult stem cells in hepatogenic media. However, most investigators have not assessed the secretion of albumin protein by cultured cells into medium. Albumin secretion is one of the most important functions of hepatocyte. In this respect, the best sources or culture condition for the differentiation of adult stem cells into functional hepatocytes have not been found yet. In the present study, we isolated adult stem cells from human eyelid adipose tissue (HAD) and successfully differentiated them into hepatocyte-like cells *in vitro*.

MATERIALS AND METHODS

1. Chemicals and Reagents

Dulbecco's modified Eagle's medium-low glucose (DMEM-LG), penicillin, streptomycin, fetal bovine serum (FBS), phosphate buffered saline (PBS), Mixture of insulin, transferrin, and selenium (ITS), and trypsin were purchased from Gibco BRL (Grand Island, NY, USA). Acrylamide, bisacrylamide and *N*,*N*,*N*',*N*'-tetramethylethylenediamine were purchased from Bio-Rad (Hercules, CA, USA). Mouse monoclonal antibodies against human albumin (ALB) and human alpha fetoprotein (AFP) were purchased from Zymed (San Francisco, CA, USA) and human cytokeratin 18 (CK18) and detection kit was from Dako (Carpinteria, CA, USA). Fibroblast growth factor (FGF)-2, FGF-4, hepatocyte growth factor (HGF) and epithelial growth factor (EGF) were purchased from Peprotech (Princeton, NJ, USA). Auroprobe^{TM}BL PLUS goat anti-mouse immunoglobulin G (IgG) and reagent A and B were purchased from Amersham (Buckinghamshire, England). Collagenase A and DNase were purchased from Roche (Rotkreuz, Switzerland). Oligo (d) T and avian myeloblastosis virus reverse transcriptase (AMV-RT) were purchased from Promega (Madison, WI, USA). Deoxyribonucleotides (dNTPs) and Taq polymerase were purchased from Fermentas (Hanover, MD, USA). RNase inhibitor was purchased from Takara (Japan). Urea assay kit was obtained from BioAssay Systems (Hayward, CA, USA). Unless specified elsewhere, all other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Human follicular fluid (hFF) was obtained from volunteers undergoing *in vitro* fertilization program with informed consents. hFF was centrifuged at 3,000 rpm for 30 min and then the supernatant was preserved at −20℃.

2. Isolation and Culture of HAD

Human adipose tissue was obtained by liposuction as discarded tissue from surgical interventions donated by volunteers with the informed consent. All experiments

were approved by Institutional Review Boards of Seoul Women's University. Fat was washed in PBS to remove blood and cellular debris and dissected into small pieces. Adipose tissue fragments were digested with 0.075% collagenase I in PBS at 37℃ for 30 min with intermittent shaking. Thereafter, they were transferred to DMEM-LG containing 10% FBS and then centrifuged at 3,000 rpm for 5 min. Supernatants were discarded and pellets were re-suspended in DMEM-LG. After several washes with DMEM-LG, the dispersed stromal cells were plated on 25mL-size culture flask (Nunc, Rochester, MN, USA) in DMEM-LG containing FBS. The medium was changed twice a week after 7 days from the primary culture. When achieving confluence, cells were treated with 0.125% trypsin and 1 mM ethylenediamine tetra acetic acid (EDTA) for 3 min. Released cells were collected and subcultivated again. Morphologically homogeneous population of fibroblast-like cells, namely HAD, was obtained after 1 or 2 sub-cultures.

3. Hepatic Differentiation

The surface of the 48-well culture plates (BD Falcon, NJ, USA) was coated with 10 μg/mL human fibronectin diluted in PBS. The plates were left overnight at 37℃, and then were washed with PBS three times. HAD were cultured in various biphasic culture conditions. In the first step of initiation, 5×10^3 cells were initially cultured in a initiation medium (IM) consisting of DMEM-LG, 10% FBS, 40 ng/mL HGF, 20 ng/mL OSM, 1 μM dexamethasone (Dex), 1x ITS, 100 μM ascorbic acid 2-phosphate (Asc) and 10 ng/mL EGF. After culture for 6 days, cells were divided into four groups for the second step of maturation. A group of HAD was cultured for 15 days in IM and the other group was cultured for 15 days in IM containing 1% DMSO, DMSO and 20 ng/mL FGF-2 or DMSO and FGF-4.

4. RT-PCR

Total RNA was isolated using Trizol method. They were allowed to stand at 65℃ for 5 min and was quantitated spectrophotometrically. Purity of RNA was assessed by measuring the ratio of absorbance at 260 nm to that at 280 nm (>1.8). RT-PCR was carried out using a GeneAmp PCR system 2400 (Perkin Elmer, Boston, USA). Total 7.5 μg (1 μg/μL) RNA was reverse-transcribed using the following RT mixture: 25 mM $MgCl_2$, 10×PCR buffer, 2.5 mM dNTPs mixture, 0.5 mg/mL oligo $(d)T^{15}$, 40 U RNase inhibitor and 20 U AMV-RT. RT reaction was carried out for 60 min at 42℃. RT products were directly used for PCR or stored at −20℃. PCR was performed using the following PCR mixture: 25 mM $MgCl_2$, 10× PCR buffer, 2.5 U Taq polymerase, 100 pM sense and antisense primers. Hepatic-relate gene primer (Table 1) used for the RT-PCR analysis. Amplification was performed for 35 cycles at a denaturing temperature of 94℃ for 30 sec and an extension temperature of 72℃ for 30 sec. Annealing temperature was set depending on the primer (Table 1). The PCR products were mixed with 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose) and separated on 2% agarose gels. After the gel was stained with ethidium bromide, DNA signals on the gel were imaged under ultraviolet light using a Bioprofile image analysis system (Viber Lourmat, Marne la Vallee, France).

5. Immunocytochemical Analysis

For the immunocytochemical analyses, cells were washed with PBS three times and fixed with 4% paraformaldehyde in PBS for 1 h at 4℃. They were rinsed with PBS and then permeabilized with 0.5% Triton X-100 in PBS for 10 min. After several washing, cells were incubated in 3% hydrogen peroxide (Dako, CA, USA) for 15 min to quench the endogenous peroxidase activities. They were rinsed with Tris buffer consisting of 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.6) and 0.05% Tween 20 and incubated in blocking solution containing 2% BSA for 1 h at room temperature. Cells were then incubated with mouse monoclonal antibodies against ALB (1:100), CK18 (1:50) and AFP (1:200) for 17 h at 4℃. For the secondary

Table. 1 Primers used for the RT-PCR analysis and expected size of PCR products

Gene	Primer sequence	Size (bp)	Temp. (℃)	Reference
ALB	5'-TTGGGAGAAGAAAATTTCAA-3' 5'-TATACCTTTTAGCAAAGAAAAGGA-3'	445	49	NM_001875
AFP	5'-GTGCTGCACTTCTTCATATGC-3' 5'-TGACAGCCTCAAGTTGTTCC-3'	218	54	NM_001134
α1-AT	5'-ACTGTCAACTTCGGGGACAC-3 5'-CCCCATTGCTGAAGACCTTA-3'	517	62	NM_000295
TTR	5-AACCAGTGAGTCTGGAGAGC-3 5-TGCCTGGACTTCTAACATAGC-3	358	55	NM_000371
CK18	5-GAGATCGAGGCTCTCAAGGA-3 5-CAAGCTGGCCTTCAGATTTC-3	357	57	NM_000224
GS	5-GTCAAGATTGCGGGGACTAA-3 5-TACGATTGGCTACACCACCA-3	397	55	BC_031964
CPS-1	5-CAAGATTCCTTGGTGTGGC-3 5-TCTGATGGAAGAGAGGCTGG-3	158	57	NM_001875
PEPCK	5-AACGCCATGGCTACAATCC-3 5-AGGTAGCTCCGAATGTCACG-3	752	60	Seiji et al., 2004
TDO	5-AGGTCAATGATAGCATCTGCC-3 5-TGTCATCGTCTCCAGAATGG-3	471	53	Seiji et al., 2004
CYP3A4	5-TGTATGAACTGGCCACTCACC-3 5-TAGCTTGGAATCATCACCACC-3	244	56	NM_017460
HNF1α	5-CAGTCTTCTTACTTGGAACTGAA-3 5-CTTGGGAACAAATACAGGAA-3	444	54	NM_000545
HNF4α	5-GAGCAGGAATGGGAAGGATG-3 5-GGCTGTCCTTTGGGATGAAG-3	205	60	NM_178849
c-MET	5-CAATGTGAGATGTCTCCAGC-3 5-CCTTGTAGATTGCAGGCAGA-3	559	55	Seung et al., 2005
CK19	5-CGAACCAAGTTTGAGACGGAAC-3 5-CCGCTGGTACTCCTGATTCTGC-3	600	62	Ines et al., 2006
CX32	5-GGCGTGAACCGGCATTCTAC-3 5-ACAACAGCCGGAACACCACG-3	400	61	Ines et al., 2006
Transferrin	5-GTGGCCTTTGTCAAGCA-3 5-CTCCATCCAAGCTCATG-3	565	52	Ines et al., 2006
GAPDH	5-ACAACTTTGGTATCGTGGAA-3 5-AAATTCGTTGTCATACCAGG-3	456	53	NM_002046

ALB, albumin; AFP, alpha fetoprotein; α1-AT, α1-antitrypsin; TTR, transthyretin; CK18, cytokeratin 18; GS, glutamine synthase; CPS-1, carbamoyl phosphate synthetase 1; PEPCK, phosphoenolpyruvate carboxykinase; TDO, tryptophan 2,3-dioxygenase; CYP3A4, cytochrome P450 3A4; HNF1α, hepatic nuclear factor 1α; HNF4α, hepatic nuclear factor 4α; c-MET, hepatocyte growth factor receptor; CK19, cytokeratin 19; CX32, connexin 32; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

antibody treatment, they were incubated with biotinylated goat anti-mouse IgG and anti-rabbit IgG for 20 min at room temperature. Then cells were incubated with horseradish peroxidase-conjugated streptavidin (Dako, CA, USA) for 20 min at room temperature. Immunoreactivity was visualized utilizing the 3,3'-diaminobenzidine tetra-

hydrochloride. Finally they were counterstained with Mayer's Haematoxylin and observed under a light microscope (LSM410; Carl Zeiss, Oberkochen, Germany).

6. PAS Staining for the Glycogen Granules

HAD were cultured onto 8-well chamber slides (Nunc, NY, USA). After culture, they were fixed in 10% formalin in methanol for 30 min at room temperature and rinsed with PBS. They were permeabilized with 0.1% Triton X-100 for 10 min, oxidized in 1% periodic acid for 10 min and rinsed three times in deionized H_2O (dH_2O). After treatment with Schiff's reagent for 30 min, they were washed two times with 0.5% sodium bisulfite in 0.05 N HCl for 10 min. Cells were counterstained with Haematoxylin for 3 min, rinsed in dH_2O and observed for the staining using a light microscope.

7. Immunoblotting Analysis for the Secreted Albumin

After differentiation culture, cells were transferred to a starvation medium of serum-free DMEM-LG and cultured again for 20 h. The starvation medium was mixed with an equal amount of 2x sample buffer consisting of 0.125 M Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 20% glycerol and 0.004% bromophenol blue, and then boiled at 95℃ for 5 min. Samples of 5 μL were separated by electrophoresis on 8% SDS-polyacrylamide gel. To hydrate polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore), they were soaked in absolute methanol for 15 sec, in distilled water for 2 min and then equilibrated in transfer buffer for 5 min. Proteins on the gel were electrotransferred onto a PVDF membrane for 60 min at 4℃ at 100V. membranes were incubated at 37℃ for 1 h in a washing buffer containing 0.8% NaCl, 0.02% KCl, 0.14% $Na_2HPO_4 \cdot 2H_2O$, 0.02% KH_2PO_4, 0.2% Tween 20, 10mM sodium azide and 5% BSA. To saturate non-specific binding sites, membranes were then incubated for 1 h in washing buffer containing 1% normal goat serum. Then they were incubated with 2 μg/mL rabbit polyclonal antibody against human albumin for 1 h and then with 1 : 100 diluted gold-labeled goat anti-rabbit IgG. After reaction, the signal was visualized using an IntenSE BL kit (Amersham, Buckinghamshire, England) according to the manufacturer's instructions.

8. Urea Assay

After culture, cells were incubated in serum-free DMEM-LG containing 5 mM NH_4Cl for 20 h in 5% CO_2 at 37℃. The concentration of urea in the conditioned medium was measured by a colorimetric assay kit (Heyward, CA) according to the manufacturer's instructions. Dilutions of a stock solution of urea were used to create a standard curve. Samples from experimental groups were analyzed in duplicate for each condition. The absorbance of the basal medium was subtracted from the absorbance of each test sample to obtain the final absorbance value for determining urea concentrations from the standard curve.

RESULTS

1. Morphological Change of HAD during Differentiation

During culture in control medium for the maintenance without differentiation induction, the morphological appearance of HAD remained fibroblastic (Fig. 1A). However, they began to change morphology during culture in IM and after 14 days, some of HAD showed round-shape typical of hepatocyte (Fig. 1B). Upon cultivation for 14 days in IM containing DMSO, about half of them showed into round-type morphology (Fig. 1C). Addition of FGF-2 to the above medium increased the number of HAD appearing as round type. However, addition of FGF-4 instead of FGF-2 was the most effective to induce the appearance of round-type morphology such that about 80% or more of HAD showed round-type cells (Fig. 1D and 1E).

2. Gene Expression Profile

Of the human hepatocyte-related genes, examined in the present study HAD exhibited differential gene expression profiles depending on the culture condition (Fig. 2). HAD

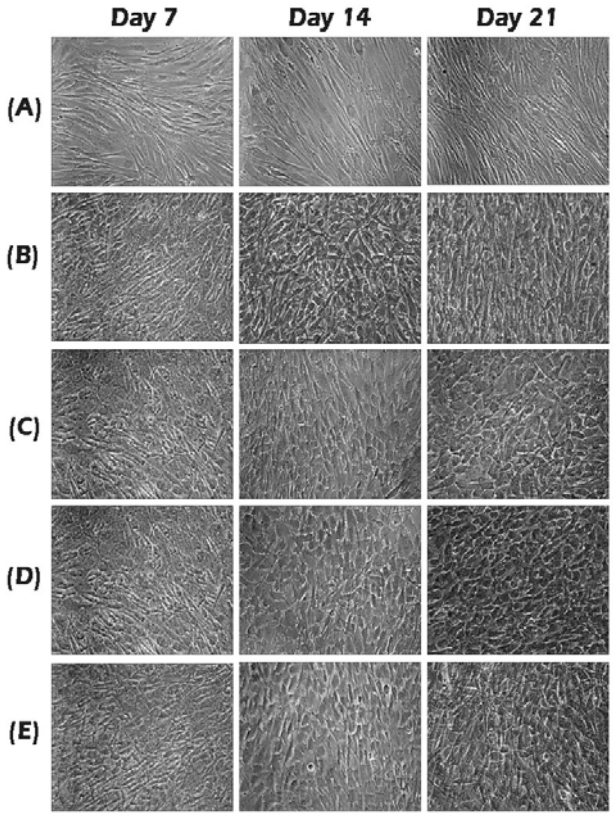

Fig. 1. Morphology of the HAD at 4th passage cultivated for 21 days *in vitro*. A, HAD cultured in the control medium without differentiation induction; B, HAD cultured in IM; C, HAD cultured in IM containing DMSO; D, HAD cultured in IM containing DMSO and FGF-4; E, HAD cultured in IM containing DMSO and FGF-2. ×100.

maintained in the control medium spontaneously expressed mRNAs of ALB, AFP, α1-AT, CK18, GS, CYP3A4, HNF4α, and CK19 genes. These undifferentiated HAD did not express genes of TTR, CPS-1, PEPCK, TDO, HNF1α, c-MET, CX32 or transferrin. However, HAD cultured in IM expressed mRNAs of PEPCK, and HNF1α. HAD cultured in IM containing DMSO expressed mRNAs of CPS-1, TDO and c-MET genes additionally. Further addition of FGF-4 to the above medium induced HAD to express additional genes of TTR and transferrin. HAD before or after culture never expressed a CX32 gene while fetal liver showed a weak expression. These results demonstrate that HAD, in terms of gene expression profiles, could better differentiate into hepatocyte-like cells *in vitro* upon culture in IM containing DMSO and FGF-4 than other culture conditions examined in the present study.

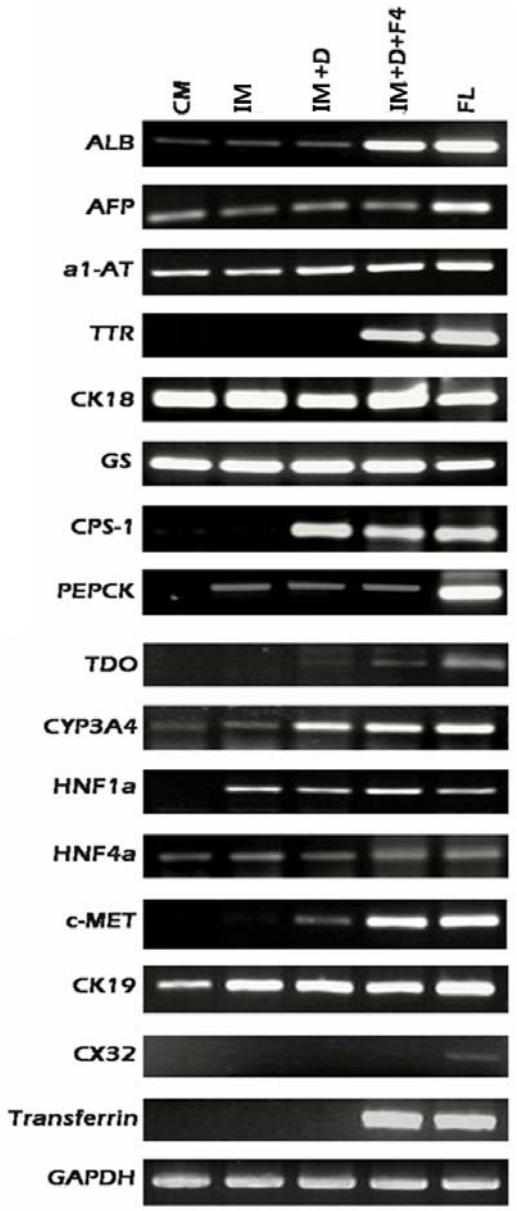

Fig. 2. RT-PCR analysis of hepatocyte-related gene expression. CM, control medium; IM, induction medium; IM+D, HAD cultured in IM containing DMSO; IM+D+F4, HAD cultured in IM containing DMSO and FGF-4; FL, mRNAs of human fetal liver as a positive control. Glyceraldehyde-3-phosphate dehydrogenase was served as an internal standard.

3. Immunocytochemical Analysis for ALB, CK18, and AFP

After 21 days of culture, immunophenotype of HAD has been analyzed for ALB, CK18, and AFP proteins. Undifferentiated HAD showed negative staining against AFP antibody and positive but weak staining against CK18 and ALB antibodies (Fig. 3a, 3b, 3c). In contrast, all groups of HAD showed distinct staining intensity against all antibodies regardless whether they were cultured in IM alone, IM containing DMSO, IM containing DMSO and FGF-4 or IM containing DMSO and FGF-2 (Fig. 3d-3o). The negative control in which HAD were reacted only with the secondary antibody, anti-mouse IgG antibody, did not show discernable staining (Fig. 3p, 3q, 3r). These observations suggest that HAD *per se* might possess inherent properties of hepatic lineage cells, and IM alone could induce HAD to differentiate into hepatocyte-like cells.

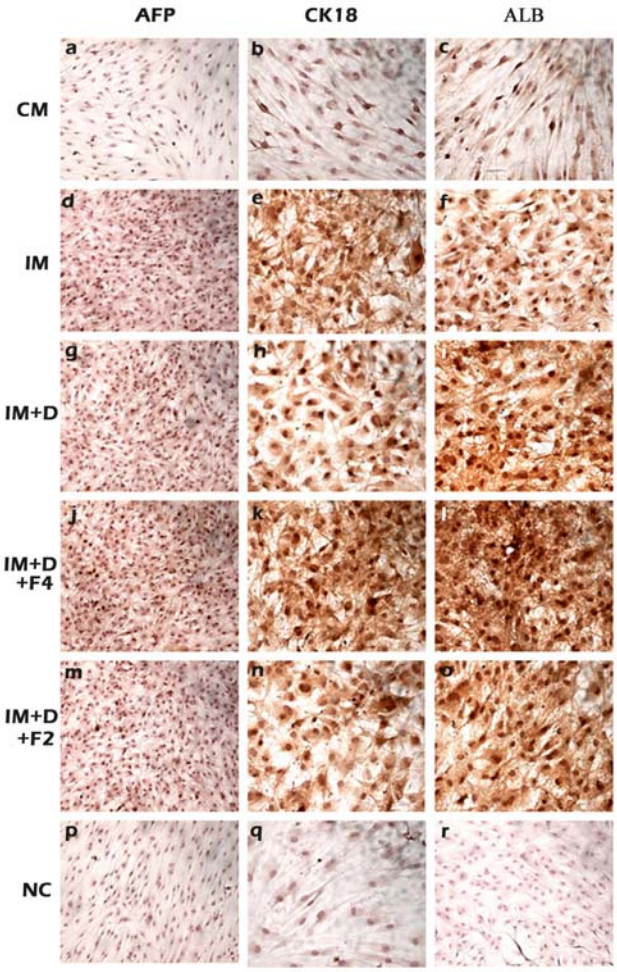

Fig. 3. Immunoreactivity of HAD against anti-human AFP, CK18, or ALB antibody. CM, HAD cultured in the control medium; IM, HAD cultured in IM; IM+D, HAD cultured in IM containing DMSO; IM+D+F4, HAD cultured in IM containing DMSO and FGF-4; IM+D+F2, HAD cultured in IM containing DMSO and FGF-2; NC, Negative control. CK18 (×200), AFP (×100), ALB (×200).

4. PAS Staining for Glycogen Storage

After culture, HAD were examined for the intracellular glycogen storage using PAS staining. Undifferentiated HAD cultivated in control medium did not show any staining indicative of intracellular glycogen storage (Fig. 4A). In

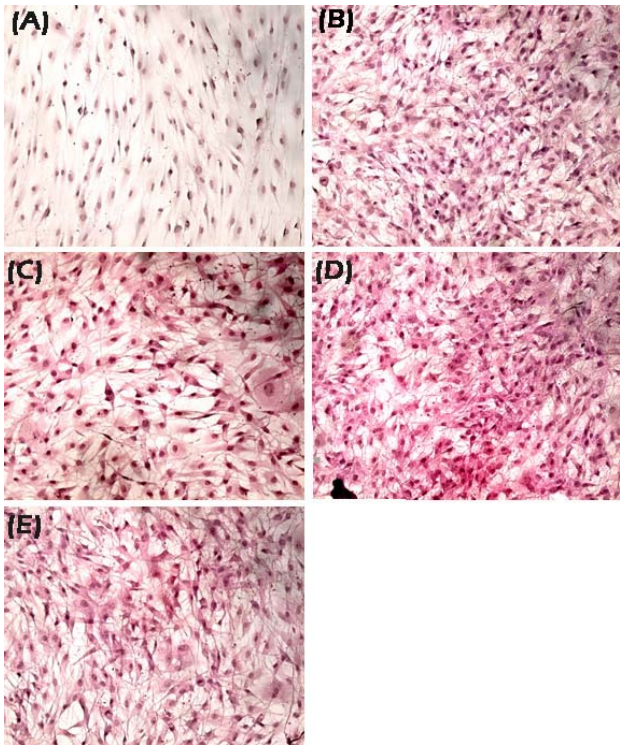

Fig. 4. PAS staining of HAD before and after differentiation induction into hepatocytes. (A) HAD cultured in the control medium; (B) HAD cultured in IM. (C) HAD cultured in IM containing DMSO; (D) HAD cultured in IM containing DMSO and FGF-4; (E) HAD cultured in IM containing DMSO and FGF-2. ×200.

contrast, HAD cultured in IM showed a distinct staining. However, HAD cultured in IM containing DMSO were more intensely stained (Fig. 4B and C). Addition of FGF-4 or FGF-2 to IM containing DMSO did not significantly enhance the staining intensity (Fig. 4D and E). These results show that DMSO could induce HAD to synthesize and store glycogen during culture *in vitro*.

5. Analysis of ALB Secretion

ALB secretion by HAD after culture were performed by immunoblot (Fig. 5). After culture for 21 days, HAD were starved overnight in DMEM-LG medium alone without any supplement. Immunoblotting analyses showed an absence of any human ALB in starvation medium from HAD cultured in the control medium. In contrast, all starvation media from HAD cultured in IM alone, IM containing DMSO, IM containing DMSO and FGF-4, or IM containing DMSO and FGF-2, showed distinct immunoreactive ALB band on the gels. Among these, however, ALB band from HAD cultured in IM containing DMSO and FGF-4 exhibited the strongest band intensity. These results showed that HAD could synthesize and secrete ALB when cultured in IM, and addition of FGF-4 might remarkably enhance the amount of ALB secretion.

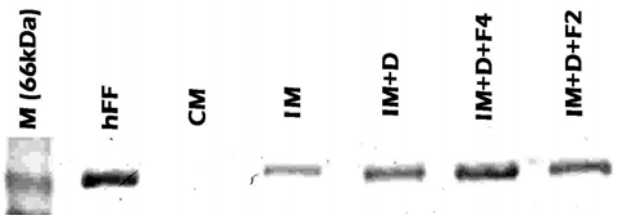

Fig. 5. Analyses of human albumin released by HAD. Immunoblot analysis of human albumin present in 2.5 μL of the above starvation medium. Five nl of hFF was used as a positive control. CM, control medium; IM, induction medium; IM+D, HAD cultured in IM containing DMSO; IM+D+F4, HAD cultured in IM containing DMSO and FGF-4; IM+D+F2, HAD cultured in IM containing DMSO and FGF-2.

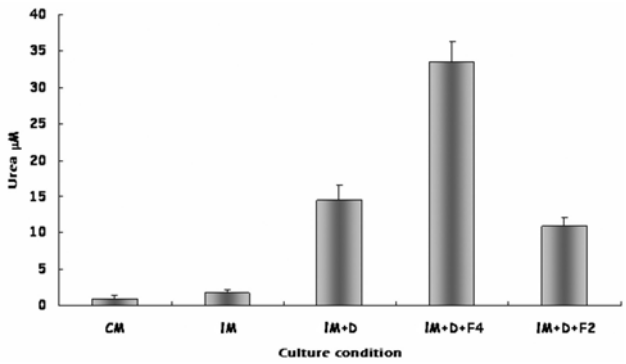

Fig. 6. Production of urea by HAD *in vitro*. After culture for 21 days in each differentiation medium, all group of HAD were stimulated overnight by DMEM-LG with 5mM NH_4Cl. The stimulated media were harvested and then assessed. Abbreviations are the same as in Fig. 1. Data are means±SEM of three independent experiments.

6. Analysis of Urea Production

Urea production by HAD was also investigated. As seen in Fig. 6, the amount of urea produced by HAD cultured in the control medium was 0.8±0.5 μM/20 h and that by HAD cultured in IM was 1.8±0.4 μM/20 h. In contrast, HAD cultured in IM containing DMSO produced urea of 14.5±2.1 μM/20 h. Further, HAD cultured in IM containing DMSO and FGF-4 produced the highest amount of urea, 33.6±2.7 μM/20 h. However, replacement of FGF-4 with FGF-2 in the last condition resulted in 10.9±1.2 μM/20 h only. These results demonstrated that HAD cultured in IM but containing both DMSO and FGF-4 could enhance the urea production than HAD of other treatment groups.

DISCUSSION

The present study demonstrates that HAD could differentiate into functional hepatocyte-like cells when cultured in IM containing both DMSO and FGF-4 *in vitro*.

HGF is well known to play an essential role in the development and regeneration of the liver (Miyazaki et al., 2002). It induces differentiation of rat bone marrow cells

into hepatocytes in culture as shown by the expression of albumin, CK 8 and 18, which are typically expressed in normal adult hepatocyte (Oh et al., 2000). Dex, a synthetic glucocorticoid supplement in hepatocyte cultures, promotes stable levels of albumin secretion and tyrosine aminotransferase induction for up to 2 months (Tong et al., 1990), and is an essential trigger for hepatic maturatio (Kinoshita & Miyajima, 2002; Zaret, 2002). During embryonic development in rodents, hematopoietic stem cells produce OSM, a member of the interleukin-6 cytokine family, enhanced cell differentiation and formation of bile canaliculi, in combination with glucocorticoid as seen in Dex-induced maturation of hepatocytes (Kamiya et al., 1999, 2001). Insulin is known to stimulate the mitosis and albumin secretion of rat hepatocytes (Kaufmann et al., 1999). Most animals synthesize Asc, an essential enzymatic cofactor for the synthesis of a variety of biological molecules and also a powerful antioxidant. Chan et al. (2005) demonstrated that endogenous Asc synthesis can confer protection against oxidative stress in rat and guinea hepatocytes. EGF plays important roles in hepatic biology (Michalopoulos et al., 2003).

Despite the presence of these additives, the lack of terminal differentiation of primary hepatocytes in culture evidences that additional signals are necessary. One of signals known to influence differentiation to hepatocytes is an extracellular matrix (ECM)-derived signal (Kinoshita & Miyajima, 2002). It has been shown that attachment of rat fetal hepatocytes was increased by ECM proteins of collagen I, fibronectin or entactin-collagen IV-laminin to a similar extent. However, cells cultured on fibronectin presented the maximal levels of expression for liver specific genes, such as ALB or AFP coincidently with an increased expression of hepatocyte nuclear factor-4, compared with those cells cultured on others (Sanchez et al., 2000).

For the optimal differentiation of HAD into hepatocyte-like cells *in vitro*, we developed a two step differentiation protocol consisting of the cell culture in IM alone for the first step of differentiation and the culture in IM containing DMSO in the presence of FGF or not, for the second step of maturation. Seo et al. (2005) reported that the addition of DMSO into differentiation medium at the first day of induction increased cell detachment from culture dishes. However, the addition of DMSO on the 7th day after differentiation induction did not show the cell detachment in the present study. DMSO added to IM enhanced ALB content and the glycogen production by HAD. DMSO has been known to maintain the functions of adult hepatocytes *in vitro* (Kamiya et al., 2002). It has been reported that nicotinamide and DMSO remarkably enhanced the emergence of small hepatocytes, and that OSM also synergistically enhanced the selective growth of small hepatocytes and inhibited the growth of blood cell populations (Sakai et al., 2002). Although OSM alone had very weak effect on hepatocyte functions, ALB secretion and cytochrome P450 capacity were greatly enhanced when treated with nicotinamide or DMSO.

FGFs play an essential role in patterning to induce hepatic fate. FGF-2 induces the foregut endoderm to the hepatocyte lineage that is required to induce a hepatic fate (Jung et al., 1999). However, a previous study showed that FGF-2 failed to induce hepatic differentiation of human multipotent adult progenitor cells *in vitro* (Schwartz et al., 2002), similar to the present results. FGF-4 is important in the initial endoderm patterning and may play a role in endoderm specification (Shi et al., 2005). Its expression in primitive streak-mesoderm can induce the differentiation of mouse endoderm in a concentration-dependent manner (Wells & Melton, 2000). FGF-4 alone or in combination with HGF was also shown to induce the hepatic differentiation of human multipotent adult progenitor cells *in vitro* (Schwartz et al., 2002).

ALB is the major protein synthesized by hepatocytes. Its production starts in the early stage of liver development and reaches the maximum level in the adult liver (Kamiya et al., 2001). Since hepatocytes consistently release ALB into the blood stream, it is critical to examine its presence in the culture medium to demonstrate the hepatic diffe-

rentiation of targeted cells. Most studies on hepatic cell differentiation have demonstrated the ALB synthesis using only immunocytochemical analysis. Little has proved its secretion into the extracellular space, which can be demonstrated by immunoblotting analysis. The present study demonstrated synthesis and secretion of ALB by HAD using both immunocytochemical and immunoblotting analyses.

Taken together, we have succeeded to differentiate HAD into functional hepatocyte-like cells using our own two-step culture method. Based on these observations, it is concluded that human eyelid adipose-derived stem cells could readily differentiate into functional hepatocyte-like cells *in vitro* and thus could be a source of therapeutic cells for the human liver diseases.

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