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Production of hepatocyte-like cells from human umbilical vein mesenchymal stem cells

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Abstract

The human umbilical vein, as a readily available stem cell source, is a good alternative to harvest mesenchymal stem cells. Human umbilical cord vein mesenchymal stem cells have recently been isolated and have demonstrated the ability to differentiate into various cell types such as fat, bone, cartilage and neuronal cells. In this study, we have investigated whether human umbilical cord vein mesenchymal stem cells are also able to differentiate into hepatocyte-like cells. Hepatic differentiation was performed with a 2-step protocol and the use of hepatocyte growth factor and oncostatin M for cell culture. During four weeks of induction, most cells displayed a cuboidal morphology. Immunological analysis indicated that umbilical cord vein mesenchymal stem cells-derived hepatocyte-like cells expressed liver-specific protein markers such as albumin and cytokeratin-18. The hepatocyte-like cells also displayed several characteristics of hepatocytes, including expression of transthyretin, glucose 6-phosphatase, cytokeratin-8,18, alpha-fetoprotein, hepatocyte nuclear factor-3β and albumin. The result of indocyanine green cell uptake, as a test substance to evaluate hepatocyte-like cell function, was positive for differentiated cells. Glycogen storage was examined by periodic acid-Schiff staining. Accumulation of intracellular glycogen was detected in the hepatocyte-like cells. Based on these observations, we have concluded that umbilical cord vein mesenchymal stem cells are endowed with hepatogenic potential and may provide a stem cell source to be used as cell therapy for liver diseases.

Key words

UVMSCs, Differentiation, Hepatocytes, ICG Uptake

Introduction

Mesenchymal stem cells (MSCs) are a group of clonogenic cells present among the bone marrow (BM) stroma that are capable of multilineage differentiation into various cell types of mesodermal origin (Deans and Moseley, 2000). MSCs can be obtained from BM (Friedenstein and el al., 1974), amniotic fluid (Anker and Scherjon, 2003), cord blood (Lee O.K. et al., 2004) placenta and other different tissue sources (Hans et al., 2008). Recently, instead of using cord blood, Romanov et al. (2003)

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and Covas et al. (2003) have been able to obtain MSCs from cells detached from the umbilical vein. Recently, we have shown the expression of several self-renewal regulator genes as well as SSEA-4, an embryonic stem cell surface marker, in human umbilical cord vein mesenchymal stem cells (UVMSCs) (Kermani et al. 2008). In spite of many similarities, human MSCs from different origins don't show similar differentiation potential, but there is the growing interest in using these differently sourced cells in regenerative medicine (Augello et al., 2007; Gerdoni et al., 2007; Martin-Rendon et al., 2008).

Researchers have considered the plausible use of stem cells for treating liver diseases (Fox et al., 1998). Although the liver has a high proliferative power, many diseases can damage its regenerative potential. Recently, researchers have considered the possibility of producing hepatocytes from different stem cell sources. Various studies have investigated the differentiation ability of stem cells to liver cells with the use of embryonic stem cells (Baharvand et al., 2006), hepatic stem cells (Golding et al., 1995), a mononuclear cell fraction of BM (Petersen et al., 1999), multipotent adult progenitor cells (Schwartz et al., 2002) and hematopoietic stem cells (Lagasse et al., 2000).

Here we report the capability of UVMSCs to differentiate into functional hepatocyte-like cells in vitro.

Materials and methods

Isolation and culture of UVMSCs

Isolation of UVMSCs was performed as previously described. Briefly, human umbilical cords were obtained after normal term deliveries of healthy infants and brought to the laboratory in Hank's balanced salt solution (HBSS, Gibco, Paisley, United Kingdom) that contained 300 U/mL penicillin, 300 μg/mL streptomycin, 150 μg/mL gentamicin, and 1 μg/mL Fungizone. Cords were processed within 6–12 h. After disinfection in 75% ethanol for 30 s, the umbilical cord veins were washed twice with HBSS that contained 100 U/mL heparin. The distal ends were clamped and veins were filled with 0.1% collagenase type IV (Sigma, St. Louis, MO). After clamping the proximal ends, umbilical cords were incubated at 37°C for 20 min. Veins were washed with phosphate buffered saline (PBS; Gibco) and isolated cells were collected and cultured in low-glucose DMEM (Dulbecco's modified Eagle's medium-low glucose) supplemented with 10 ng/mL epidermal growth factor (EGF; Sigma), 20 ng/mL basic fibroblastic growth factor (bFGF; Sigma), 15% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). UVMSCs were isolated by their ability to adhere to a plastic surface.

Passage 5 UVMSCs were allowed to differentiate for 28 days in differentiation medium, which was changed every three days. Differentiated UVMSCs were dissociated with 0.05% trypsin and 0.04% ethylenediaminetetraacetic acid (EDTA; Gibco, Brl, Berlin, Germany) for 5 min, centrifuged, and replated in differentiation medium in 3 cm tissue culture dishes. They were grown to confluence, serially passaged, and expanded in differentiation medium.

Culture of HepG2 cells

Human hepatoma cell line, HepG2, was obtained from Pasteur Institute, Tehran, Iran and cultured in RPMI medium (Roswell Park Memorial Institute medium) supplemented with 10% FBS. We used this cell line as a positive control for differentiated hepatocyte-like cell analysis.

Hepatic differentiation of UVMSCs

Hepatic differentiation was performed by using a 2-step protocol with sequential addition of growth factors and cytokines. Cells in passage 5 were plated on fibronectin-coated dishes at a density of 1.5×10^4 cells/cm^2 in DMEM-LG (Invitrogen, Carlsbad, California) supplemented with 15% FBS, 20 ng/mL bFGF, 10 ng/mL EGF and grown to 60%-80% confluence. Cells were treated with initiation medium for two weeks that contained proliferative medium supplemented with 0.5 μmol/l dexamethasone (Sigma), 1x ITS+ Premix (BD Biosciences), 50 ng/mL hepatocyte growth factor (HGF; R&D Systems, Minneapolis, MN), 10 ng/mL EGF, and 20 ng/mL bFGF. Initiation medium was replaced by maturation medium for another two weeks. Maturation medium consisted of proliferative medium and contained 0.5 μmol/l dexamethasone, 10 ng/mL EGF, 20 ng/mL bFGF, 1x ITS+ Premix and 50 ng/mL oncostatin M (OSM, Sigma). Differentiated liver-like cells were subcultured every four to five days until the end of experiment.

RNA extraction and RT-PCR analysis

Total RNA was isolated using the RNX plus Kit (CinnaGen, Tehran, Iran), according to the manufacturer's recommendations. One μg of total RNA was reverse transcribed into DNA using AMV reverse transcriptase and a random hexamer primer (RT-PCR Kit; Bioneer, Daejeon, South Korea). RT-PCR was performed in a 50 μl reaction mixture using a thermal cycler (Mastercycler; Eppendorf, Hamburg, Germany). cDNA was then subjected to PCR amplification with a PCR kit (Bioneer) and primer sets that were specific to the genes of interest (Table 1). The PCR conditions were initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 45 s at 53-58°C (depending on primers) and extension for 60 s at 72°C. The reaction was followed by a final extension step for 10 min at 72°C. The expression of beta-2 microglobulin mRNA, a constitutively and ubiquitously expressed gene, served as an internal control in all RT-PCR reactions. The PCR products were separated on a 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and photographed on a UV transilluminator (Uvidoc, Cambridge, UK).

Immunofluorescence analysis

Cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Fixed cells were permeabilized and blocked for 45 min in PBS that contained 0.01% Triton X-100 (Sigma) and 10% serum. Then differentiated cells were incubated with mouse monoclonal anti-albumin (anti-ALB, 1:50, R&D Systems) and

Table 1 – Primers and PCR conditions for RT-PCR analysis of hepatocyte-like cells differentiated from UVMSCs.

Gene	Primer Sequence (5′ - 3′)	Length (bp)	Gene bank code
Albumin (ALB)	R: TCAAGTGTGCCAGTCTCCAA F: TGGCAAGTCTCAGCAGCA	459	NM_000477
Alpha-fetoprotein (AFP)	R: GCCAAAGTGAAGAGGGAAGAC F: GCAGACAATCCAGCACATCTC	494	NM_001134
Beta-2 mMicroglobulin (β2Mm)	R: CACGGCAGGCATACTCATC F: GTTTCATCCATCCGACATTG	199	NM_004048
Cytokeratin-18 (CK-18)	R: ACAGTCTGCTGAGGTTGGA F: CAGACACCACTTTGCCATC	412	NM_000224
Cytokeratin-8 (CK-8)	R: TGCCTCTACCATGTCCATCA F: TGGTCTCCAGCATCTTGTTCT	374	NM_002273
Glucose 6-phosphatase (G6P)	R: GCAGAAGGACAAGACGTAGAAGA F: GCTGAATGTCTGTCTGTCACGAA	494	NM_000151
Hepatocyte growth factor receptor (c-Met)	R: TGTGTTGTCCCGTGGCCATTTG F: TGCGATCGGAGGAATGCCTGAG	307	NM_000245
Hepatocyte nuclear factor- 3β (HNF3β)	R: TGCAACACCGTCTCCCCAAAGT F: CCACCACCAACCCCACAAAATG	294	NM_021784
Transthyretin (TTR)	R: GTGACGACAGCCGTGGTGGAA F: GGTGAATCCAAGTGTCCTCTGAT	352	NM_000371

rabbit monoclonal anti-cytokeratin (CK) 18 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4ºC. The samples were then incubated for 45 min at 37ºC with FITC-conjugated secondary antibody against mouse (for ALB, 1:100; Abcam, Cambridge, UK) or TRITC-conjugated secondary antibody against rabbit (for CK-18, 1:100; Abcam). PBS-containing serum was used for washing the specimens between antibody incubations. Cover slips were mounted in mounting medium and immediately examined under an inverted fluorescent microscope (TE-300; Nikon, Tokyo, Japan).

Indocyanine green cellular uptake analysis

Indocyanine green (ICG, Acros Organics, Geel, Belgium) was added to the cell culture media of differentiated, undifferentiated and positive control cells at a final concentration of 1 mg/mL. Cells were incubated at 37°C for 30 min and then rinsed with PBS. After the dish was rinsed three times with PBS, the cellular uptake of ICG was examined by an inverted microscope.

Periodic acid Schiff (PAS) staining

Glycogen storage in the fixed, differentiated cells was examined using periodic acid-Schiff (PAS, Sigma) staining. The cells were oxidized in 1% periodic acid for

10 min and rinsed three times in distilled H_2O. Afterwards, cells were treated with Schiff's reagent for 10 min, rinsed in distilled H_2O for 10 min, and observed under a microscope.

Dil uptake staining

After four weeks, differentiated cells were incubated in culture maturation medium that contained 20 ug/mL DiI-Ac-LDL (acetylated low density lipoprotein, labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; Invitrogen) for 24 h at 37°C. Cells were fixed with 2% formaldehyde in PBS. After washing with PBS, cells were then washed with fresh PBS, further incubated in 20 mg/mL DAPI. Incorporation of fluorochrome-labeled LDL and DAPI was estimated in a fluorescent microscope (IX-71, Olympus, Tokyo, Japan) with a Texas-Red filter.

Results

Morphological changes in UVMSCs-derived hepatocyte-like cells

Human umbilical vein MSCs were expanded for at least 20 passages. Passage 5 UVMSCs were used for differentiation. The proliferative rate of differentiated cells gradually decreased.

During the first two weeks, UVMSCs in the presence of initiation medium for differentiation lost their fibroblastic aspect (Fig. 1A) and became broad and flat (Fig. 1C). Abundant granules appeared in the cytoplasm of UVMSCs-derived hepatocyte-like cell (Fig. 1D), which we did not see in undifferentiated cells (Fig. 1B). During the

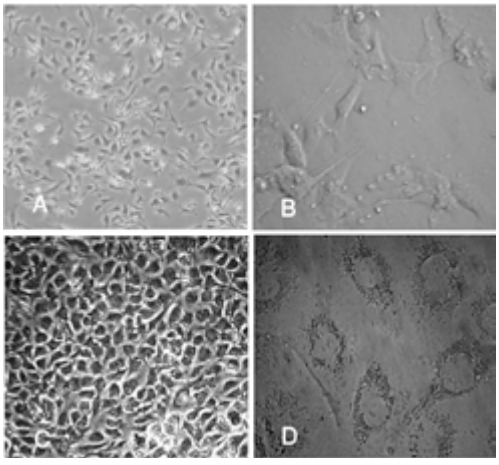

Figure 1 – Changes in cell morphology during hepatic differentiation of UVMSCs. Cultured undifferentiated cells (A, B) differentiated into round, oval or polygonal cells (C, D) upon hepatogenic condition. A, C: 100x; B, D: 400x.

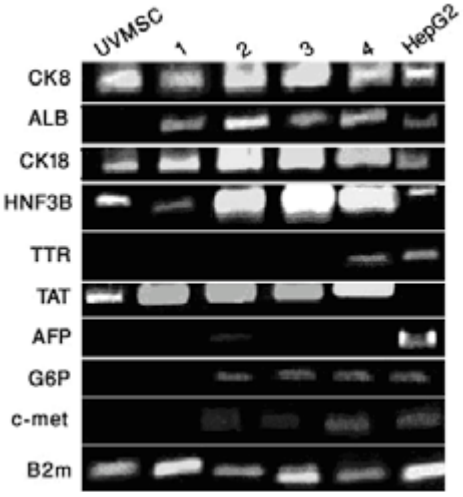

Figure 2 – RT-PCR analyses of the expression pattern of hepatocyte-specific markers during hepatic differentiation of UVMSCs. CK8: cytokeratin 8; ALB = albumin; CK18 = cytokeratin 18; HFN3B = hepatocyte nuclear factor-3β; TTR = transthyretin; TAT = tyrosine aminotransferase; AFP = alpha-fetoprotein; G6P = glucose 6-phosphatase; c-met = c-Met; B2m = β2-microglobulin. Expression of alpha-fetoprotein was noted only at the second week of induction.

third and fourth weeks of differentiation, in the presence of OSM, differentiating cells developed a cuboidal morphology characteristic for hepatic cells.

Expression profile of hepatocyte-specific genes in differentiating hepatocytes

The mRNA expression pattern of hepatocyte-specific genes was analyzed by reverse-transcription polymerase chain reaction (RT-PCR; Fig. 2). RT-PCR analysis showed the expressions of ALB by day 7, glucose 6-phosphatase (G6P) by day 14, c-Met and transthyretin (TTR) by day 28. Expressions of CK-8 and CK-18, hepatocyte nuclear factor-3β (HFN3β) and tyrosine aminotransferase were detected during differentiation at all time points and gradually increased. Alpha-fetoprotein (AFP) expression was detected only at day 14 of the experiment. Undifferentiated cells did not express ALB, G6P, TTR, c-Met nor AFP but did express low levels of CK-8 and CK-18 and HFN3β. HepG2 cells were used as positive controls.

IF analysis of hepatic protein expression

To further confirm the differentiation of UVMSCs into hepatocytes, we examined ALB and CK-18 expressions by immunofluorescence analysis. The differentiated cells stained positively for these markers (Fig. 3).

Glycogen storage as detected by PAS staining

Glycogen was detected by PAS staining in hepatocyte-like cells. Undifferentiated cells were negative with this staining (Fig. 4).

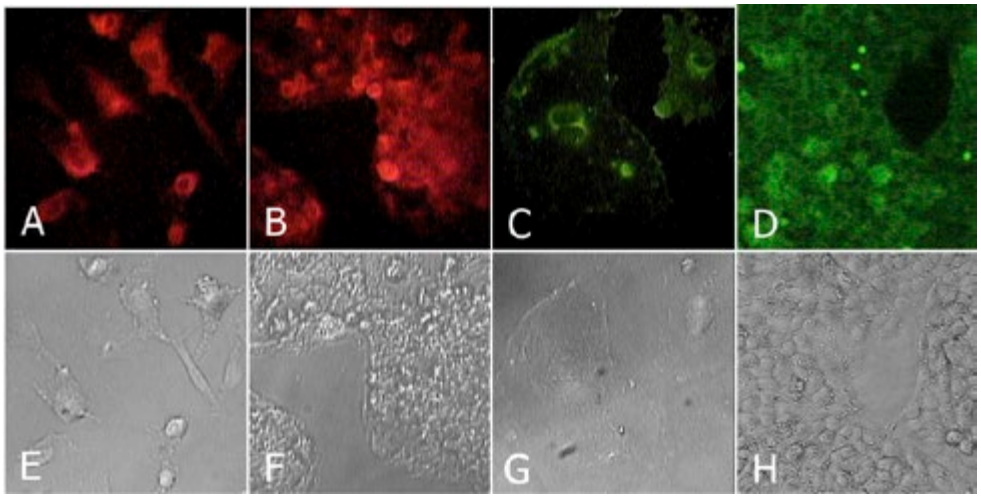

Figure 3 – Immunocytochemistry of differentiated UVMSCs into hepatocyte-like cells stained with anti-albumin and anti-cytokeratin-18 antibodies. (A, C) UVMSCs-derived hepatocyte-like cells stained for albumin and cytokeratin-18, respectively. (B, D) HepG2 cells were used as positive control. E-H show the phase contrast counterparts of A-D, respectively. 200x.

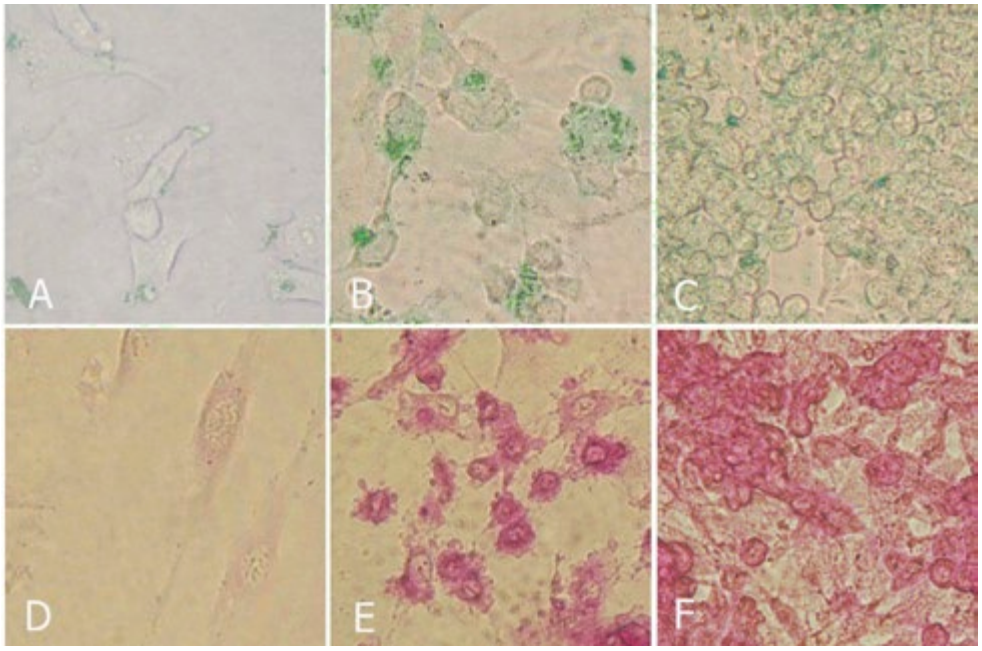

Figure 4 – Cellular uptake of indocyanine green, and glycogen storage as determined by periodic acid-Schiff staining. (A) Undifferentiated UVMSCs cells were negative for indocyanine green. (B) Most differentiated cells were positive for indocyanine green. (D) Undifferentiated UVMSCs cells were periodic acid-Schiff negative. (E) Differentiated UVMSCs cells displayed glycogen granules. (C,F) In both experiments, the HepG2 cell line was used as positive controls. 200x.

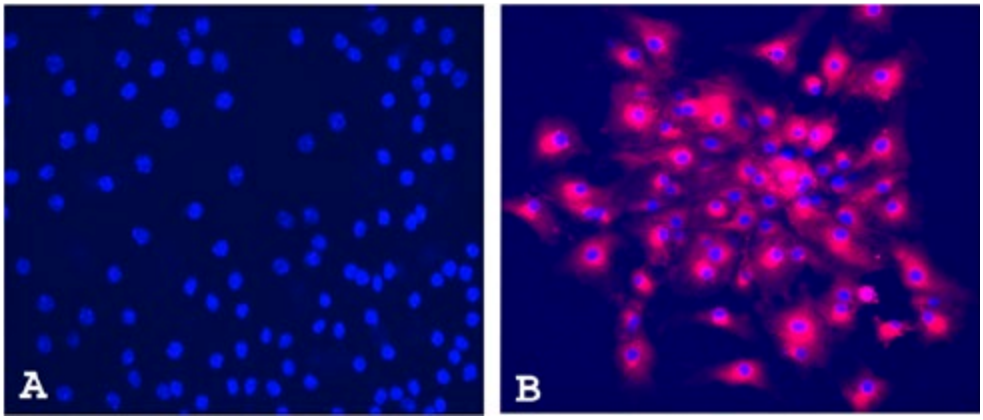

Figure 5 – LDL uptake by UVMSC-derived hepatocytes. Hepatic differentiation was performed with a 2-step protocol and the use of hepatocyte growth factor and oncostatin M. Differentiated cells were incubated with DiI-acil-LDL, fixed, and visualized with an inverted fluorescent microscope. Representative immunofluorescence micrographs are shown for undifferentiated (A, week 0) and differentiated cells (B, 4 weeks). DiI-Ac-LDL and DAPI are shown as red and blue colors, respectively. 200x.

Analysis of ICG and LDL uptake

Since ICG is clinically used as a test substance to evaluate liver function because it is eliminated exclusively by hepatocytes, we first examined the cellular uptake of ICG in a HepG2 cell line. HepG2 cells were positive for ICG, while undifferentiated cells were negative. Differentiated hepatocyte-like cells were positive for ICG.

Immunofluorescence analysis indicated that undifferentiated cells were unable to incorporate acetylated LDL, however approximately 50% of the cells that were treated for two weeks with initiation medium and another two weeks with maturation medium were able to incorporate it (Fig. 5).

Discussion

Umbilical VMSCs, as an attractive source of stem cells, were first isolated in the year 2003 (Covas et al., 2003; Romanov et al., 2003). These stem cells have the capability to differentiate into mesodermal and ectodermal lineage cells, including adipocytes, osteoblasts, chondrocytes, neurons, astrocytes and cardiomyocytes (Covas et al., 2003; Romanov et al., 2003; Kadivar et al., 2006a,b).

Recently we isolated human UVMSCs and characterized the isolated cells by evaluating the expression of certain embryonic stem cell surface markers as well as some major embryonic and adult stem cell self-renewal regulatory genes. We have shown that several self-renewal regulatory genes as well as SSEA-4, an embryonic stem cell surface marker, are expressed in UVMSCs (Kermani et al., 2008).

In this study, we investigated the hepatic differentiation of UVMSCs cells. To optimize hepatocyte formation, many different conditions were tested. We pre-incubated

the cells in serum and/or growth factor-free (EGF, bFGF) medium however the cells either died or underwent spontaneous differentiation into neural-like cells. When UVMSCs were plated at 15,000 cell/cm^2 and grown to 60% to 80% confluence, they differentiated into hepatocyte-like cells, whereas UVMSCs plated and treated at lower cell density died.

We also treated the cells with a 25 ng/mL concentration of HGF and OSM in both initiation and maturation media. Morphological and RT-PCR results indicated that the higher concentrations of HGF and OSM indeed used were required for optimal triggering of hepatogenic signal transduction systems. Finally, UVMSCs were cultured on fibronectin-coated dishes with proliferation medium and differentiated into hepatocyte-like cells by using a 2-step differentiation protocol with sequential addition of HGF, OSM and hormones (dexamethasone and insulin). mRNA expression of endodermal and hepatocyte-specific genes that included TTR, G6P, CK-8,18, AFP, hepatocyte nuclear factor-3β (HNF3β) and ALB was detected at varying stages of UVMSC differentiation. Differentiated cells were found to store glycogen and uptake ICG, which indicated the differentiation of UVMSCs into functional hepatocyte-like cells.

Hong et al. have differentiated umbilical cord blood derived MSCs into hepatocytes with the same protocol as the current study, and reported similar results (Hong et al., 2005).

Zhao et al. (2009) have recently reported in vitro transdifferentiation of umbilical cord MSCs into a hepatic lineage by using a modified 2-step induction protocol. In their study, high expressions of hepatic markers AFP and ALB were accompanied by increased production of ALB and urea in supernatant in a time-dependent manner.

Schwartz et al. (2002) have analyzed the capability of multipotent adult progenitor cells to differentiate into hepatocytes. They reported the necessity of a high cell density for hepatic differentiation. The results of this study in conjunction with those of Schwartz et al. (2002) and Hong et al. (2005) have suggested that cell–cell contact is an important factor in the hepatic differentiation process.

Talens-Visconti et al. (2006) have induced hepatic differentiation of human MSCs from adipose tissue and from BM. In their studies, cells were serum deprived for two days, pre-cultured in medium supplemented with growth factors, followed by a 2-step differentiation protocol and subsequently by the sequential addition of growth factors, cytokines and hormones. The 2-step differentiation protocol in their study has previously been used for hepatic differentiation of human BM MSCs (Lee K.D. et al., 2004).

The expression profile of hepatic-specific genes examined in our study compared with the study conducted by Hong et al. (2005) has shown that, as in that study, the level of expression for ALB and CK-18 genes gradually increased during the course of the experiment. TAT gene expression was observed in the whole study period of this experiment and to a lesser degree in undifferentiated cells. However, in the study by Hong et al. (2005) TAT gene expression was observed only during the third and fourth weeks of the experiment. In our study, c-Met gene expression was observed in the second to fourth weeks whereas in the Hong et al. (2005) study c-Met gene expression was observed only in the third and fourth weeks.

Both in this study and in a study by Lee K.D. et al. (2004) the expressions of ALB, CK-18 and G6P were detected on days 14 and 28. However, expression of TAT

in their study was seen at day 28, whereas in the current study TAT expression was seen in the whole study period of this experiment. The expression of AFP gene in this study was noted only on day 14, but in the above mentioned study it was seen at days 14 and 28.

All these findings suggest that MSCs derived from different tissue sources do not exhibit the same behavior during hepatic differentiation. This suggestion is in line with a report which demonstrated that human MSCs derived from five different tissues exhibit different differentiation potentials as manifested by the rate of differentiation and percentage of differentiated cells (Musina et al., 2006).

Because MSCs have the ability to differentiate into three lineages, these stem cells have been considered as an attractive source for tissue engineering and stem cell therapy (Romanov et al., 2003). However the number of MSCs significantly decreases with age (Rao et al., 2001; Romanov et al., 2003). It has been reported that BM-MSCs are permissive to productive HCMV infection, and that HCMV alters the function of MSCs (Smirnov et al., 2007). Sundin et al. (2006) have reported that MSCs may be susceptible to infection if infused in a patient with CMV or HSV-1 viremia.

Although umbilical cord blood is a rich source of hematopoietic stem/progenitor cells useful for clinical applications (Huss 2000; Hows 2001; Romanov et al., 2003), attempts to isolate MSCs from that blood have not always been successful (Hong et al., 2005).

Our results indicate that UVMSCs can differentiate into hepatocyte-like cells. In conclusion, the umbilical cord vein might be an easily accessible source for multipotent stem cells that are capable of differentiating toward hepatocyte-like cells in vitro.

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