



In vitro differentiation of human umbilical cord blood mesenchymal stem cells into functioning hepatocytes



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Abstract Mesenchymal stem cells (MSCs) were isolated by gradient density centrifugation from umbilical cord blood. Spindle-shaped adherent cells were permitted to grow to 70% confluence in primary culture media which was reached by day 12. Induction of differentiation started by culturing cells with differentiation medium containing FGF-4 and HGF. Under hepatogenic conditions few cuboidal cells appeared in culture on day 7. From day 21 to day 28, most of cells became small and round. The control negative cells cultured in serum free media showed fibroblast-like morphology. Urea production and protein secretion by the differentiated hepatocyte-like cells were detected on day 21 and increased on day 28. Protein was significantly increased in comparison with control by day 28. The cells became positive for AFP at day 7 and positive cells could still be detected at days 21 and 28. The cells in the control group were stained negative for AFP. The cells expressed albumin gene at the 14th day that became markedly increased at the 28th day of culture with HGF and FGF-4. No albumin expression was observed in the 7th day sample and the control. This study demonstrated that UCB-derived MSCs had the ability to differentiate into functioning hepatocyte-like cells starting from the 7th day after culturing under hepatogenic conditions and became well functioning at days 21 and 28. These data indicated that UCB-derived MSCs can be a promising source of cell therapy for intractable liver diseases.

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1. Introduction

Liver is dedicated to perform an extensive range of tissue specific functions including intermediary metabolism, detoxification of drugs or other xenobiotics as well as endogenous substrates, synthesis of plasma proteins and bile formation.¹ The principal

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cells of mature liver that carry out these functions are parenchymal hepatocytes which represent approximately 80% of total hepatic volume.²

Liver dysfunction is a major health problem in the world, and liver transplantation is the only conventional thriving treatment of end-stage liver disease.³ Unfortunately, human donor livers are very limited and are unavailable to most people worldwide who necessitate whole organ transplantation. Hepatocytes transplantation and the use of hepatocytes with extracorporeal bioartificial liver supports are other alternatives.⁴ However, the shortage of cell supplies and the long-term efficiency remains unclear which limits this strategy.³

There are requires for efficient *in vitro* models of hepatocytes developed from other sources with full metabolic function. Some cell types exhibit the potential to develop into viable hepatocytes such as embryonic stem cells (ESCs) and adult stem cells (ASCs).⁵ Studies on ESCs were limited in clinical therapeutic applications as a result of ethical controversies. On the other hand, adult SCs (like bone marrow stem cells) can be propagated in large quantities and remain capable to differentiate into various tissue types⁶ including hepatocyte lineage.⁷

Recently, human umbilical cord blood (HUCB) has been used as a rich, easily available and ethically acceptable source of stem cells (SCs). UCB can be collected from donors without invasive or painful procedure and is rarely infectious.⁸ UCB-derived cells are also considered more primitive than BM-derived cells so they represent a more appropriate source for cellular therapies.⁹

There are 2 types of stem cells in HUCB: mesenchymal (MSCs) and hematopoietic. Lately it has been reported that UCB mesenchymal progenitor cells are capable of differentiating into hepatocytes.¹⁰ Isolated MSCs from HUCB were induced to differentiate into hepatocyte-like cells under the effect of pro-hepatogenic conditions of different cytokines and growth factors including fibroblast growth factor (FGF) and hepatocyte growth factor (HGF).¹⁰⁻¹³ Transformation of the fibroblast-like cells into round epithelioid cells expressing mRNAs of albumin (ALB), and alpha-fetoprotein took place under these hepatogenic conditions^{10,11,13}. The functional capacity of the hepatocyte-like cells was assessed by albumin and urea secretion^{10,12} and the ability to integrate DiI-acetylated low-density lipoprotein.^{10,11}

The aim of this work was to study the functional activity of hepatocyte-like cells derived from HUCB-MSCs at different time points by reverse transcription-polymerase chain reaction (RT-PCR) for albumin gene expression level, immunocytochemical stain for detection of alpha-fetoprotein in the cell cytoplasm and biochemical assay of protein and urea levels secreted in the culture media.

2. Materials and methods

The study protocol was approved by the ethics committee of the Mansoura University.

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), recombinant human fibroblast growth factor (FGF)-4, penicillin/streptomycin/L-glutamine (100×), recombi-

nant human hepatocyte growth factor (HGF), Ficoll-Paque (1077), all materials were purchased from Sigma, Egypt.

2.2. Collection of umbilical cord blood

UCB samples (No = 22) were obtained from Department of Obstetrics and gynecology, Mansoura University Hospital. Samples were collected after informed consent was given from full-term elective cesarean sections before placental separation. Family history of gene disorders and maternal fever was excluded. The umbilical cord was double clamped two inches apart from infant's abdomen, wiped with 70% alcohol followed by Betadine at needle insertion site to insure sterility. The needle insertion site was just above the clamp. The blood samples were obtained in special cord blood collection bags.¹⁴

2.3. Mesenchymal stem cell isolation and culture

The collected blood processed within 6 h of collection and the samples were transported in sterile tubes and diluted with phosphate buffer saline (PBS) in concentration 1:1. Mesenchymal stem cells were isolated using Ficoll gradient density centrifugation. Ficoll was added to the sample in concentration 3:1. Ficoll-Paque was normally placed at the bottom of a conical tube, and the blood was then slowly layered above it. Centrifugation of the tubes was done at 400 RCF for 30 min. The supernatant was discarded and the puffy coat layer which contained mononuclear cells was aspirated and transferred into other tube. PBS was added for washing in concentration 1:1 and centrifuged at 2000 RPM for 5 min. The supernatant was discarded.

The suspended precipitated cell pellet was cultured in primary complete culture media (containing 20 ml DMEM – 1% L-glutamine- 10% fetal bovine serum – 1 ml antibiotic) and plated in tissue culture flasks (25 cm²). The samples were incubated in incubator (37 °C) with 5% CO₂ level and were nourished every 3 days. Feeding step was performed by discarding the old media and adding 20 ml new complete media as mentioned before.

After culturing cells for 12 days in primary culture media, cells reached 70–80% confluence. The old media was discarded and 10 ml trypsin was added to each flask for 5 min. The flasks were shaken to ensure that the cells were completely detached from the flask wall, then the content of the flasks was put in tubes to be centrifuged for 5 min at 2000 RBM. The cell pellets appeared on the wall of the tube, the supernatant was discarded, finally the cells in the media were transferred into new flasks (25 cm²) for subculture.¹⁵

2.4. Hepatogenic differentiation

To start induction of cell differentiation into hepatocytes, second- to third-passage cells, at approximately 70% confluence were treated with differentiation medium consisting of serum free DMEM supplied with 20 ng/mL HGF, 10 ng/ml FGF4 and 1% antibiotic penicillin and streptomycin 12. Feeding was carried out two times per week and hepatogenic differentiation was investigated at different time points (7th -14th -21st -28th day) after treatment with differentiation medium.

2.5. Reverse transcription–polymerase chain reaction (RT-PCR) for albumin gene

Extraction of RNA was done from 1×10^6 MSCs differentiated cells using RNEasy (Sigma) as per the manufacturer's instructions. The reverse transcription of mRNA to complementary DNA was done using Advantage RT-for-PCR (Sigma) as per the manufacturer's instructions.

The resulting cDNA was amplified by the use of ABI GeneAmp PCR System 2400 (Sigma) under the following conditions: For human albumin gene (50-CTTGAATGT GCTGATGACAGG, 50 –GC AA GT CA GC AGGCATCT CATC) amplification: initial denaturation (94 °C for 5 min),

annealing (35 cycles at 94 °C for 90 s), extension (57 °C for 2 min) and final extension (72 °C for 3 min). For GAPDH gene (50-GT CTTCTCCACCATGGAGAAGGCT, 50-CA TG CC AG TGAGCT TC CCGTTCA) amplification: denaturation at 95 °C for 300 s, annealing (30 cycles at 94 °C for 30 s), extension (55 °C for 30 s) and final Extension (72 °C for 60 s).

Detection of amplification products: The products of PCR were subjected to agarose gel electrophoresis using 2% agarose gel containing 2 μ L of 10 mg/1 ml ethidium bromide (Sigma), using 1 \times Tris–Borate-EDTA Buffer (TBE Buffer) for one hour at 70 V.

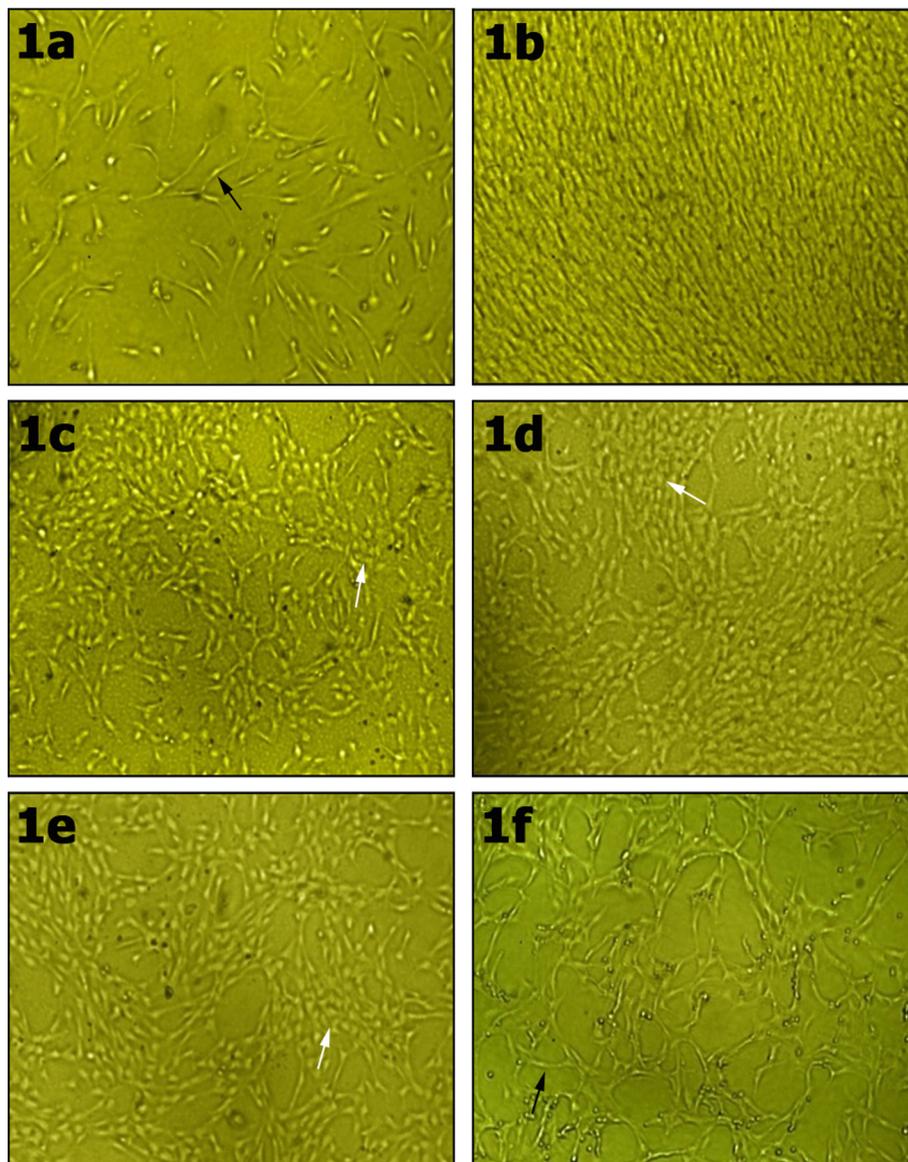


Figure 1 Human umbilical cord blood derived mesenchymal stem cells (UCB-MSC) in primary culture media on day 4 with fibroblast like morphology (black arrow) (1a). Cells in primary culture media at day 12 were extensively expanded and overlapped in some areas (1b). After treatment with FGF4 and HGF for 7 days, few rounded cells appeared (white arrow) (1c). The number of cuboidal or round cells (white arrow) increased after 14 days of culture under hepatogenic conditions (d). By day 21 to day 28 of culturing under hepatogenic conditions, many cells were rounded (white arrow) (e). The cells of the control negative group were fibroblast-like (black arrow) (f). Phase contrast microscope $\times 100$.

2.6. Assay of protein level and urea in the culture media at different time points

Samples were obtained from cell culture media under hepatogenic conditions at different time points (7th -14th -21st -28th day). Serum free medium was used as a negative control. Collection and investigations of samples were done for urea and protein levels.¹⁶

2.7. Immunocytochemical study for alpha fetoprotein (AFP)

Cells were fixed with 95 mL/L ethanol for 10 min at 4 °C, and incubated with blocking solution for 10 min to block endogenous peroxidases. Incubation with the primary antibody (anti-AFP) was done for 30–60 min at room temperature. The slides were then incubated with enzyme conjugate followed by DAB (Power-Stain TM 1.0 Poly HRP DAB Kit, Genemed Biotechnologies, Inc.) according to the manufacturer's instructions. The slides were then counterstained with hematoxylin, dehydrated and mounted. Positive reaction appeared as brown cytoplasmic stain.

2.8. Statistical analysis

The data of protein and urea assay were expressed as mean \pm SD. The statistical software SPSS12.0 was used. The results were analyzed by *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation and culture of MSCs from UCB

Mesenchymal stem cells (MSCs), obtained by gradient density centrifugation, were heterogeneous during the first few days. Spindle-shaped cells were observed on the 4th day adherent to the bottom of culture flasks while many round cells were floating in the medium. Changing the medium progressively decreased the floating cells till their disappearance.

3.2. Expansion and hepatogenic differentiation of HUCB-MSC

Human umbilical cord blood derived mesenchymal stem cells (HUCB-MSC) were permitted to grow to 70% confluence in primary culture media. After 4 days of culture in primary culture media, the adhered cells were fibroblast-like and few in number (Fig. 1a). By day 12, the primary cultured cells reached a 70–80% confluence and the cells extensively expanded and overlapped in some areas (Fig. 1b).

After that, cells were subcultured and differentiation was started by culturing cells with differentiation medium containing FGF-4 and HGF. Under hepatogenic conditions with FGF4 and HGF, cells did not expose any change compared to the control before the 7th day of treatment. On day 7, few cuboidal or round cells appeared in culture (Fig. 1c). After 14 days, the number of cuboidal cells increased in the culture (Fig. 1d). From day 21 to day 28, most of cells became cuboidal or round (Fig. 1e). The control negative cells cultured in serum free media containing DMEM, 1% L-glutamine, penicillin (100 U/mL) and

streptomycin (100 U/mL), showed fibroblast-like morphology and no round shaped cells were found (Fig. 1f).

3.3. Urea and protein assay

Urea production and protein secretion by the differentiated hepatocyte-like cells were detected at different time points during differentiation. Under hepatogenic conditions by treatment with FGF-4 and HGF, urea produced by MSCs was significantly higher compared to control on day 21 and increased on day 28 (Fig. 2). Protein was significantly increased in comparison with control by day 28 (Fig. 3).

3.4. Immunocytochemistry staining for AFP

After culture with both HGF and FGF-4, the cells were positive for AFP at different time points 7, 14, 21 and 28. The

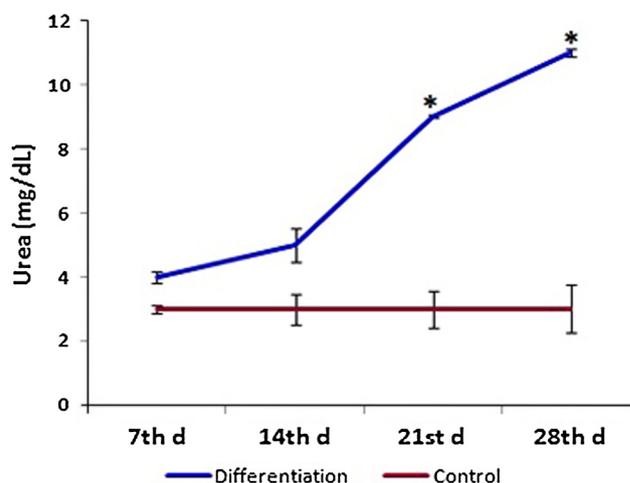


Figure 2 The level of urea (mean \pm SD) in the culture media was significantly (*) increased on day 21 and day 28. The control level showed stability. $p < 0.05$.

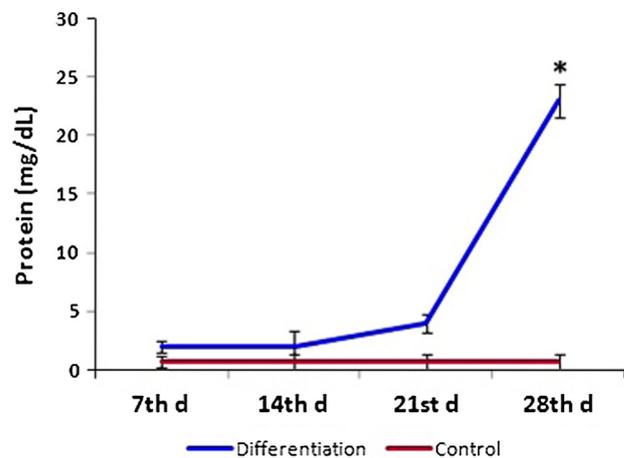


Figure 3 The level of protein (mean \pm SD) in the culture media significantly (*) increased at day 28. The control level showed stability. $p < 0.05$.

results showed that the AFP appeared by day 7 and could still be detected by day 21 and day 28 (Fig. 4a–d). The cells in the control group were stained negative for AFP (Fig. 4e).

3.5. Gene expression study using reverse transcription polymerase chain reaction (RT-PCR)

The expression of GAPDH as a control gene did not differ in between the control and other samples under hepatogenic conditions indicating valid mRNA in all specimens (Fig. 5).

The albumin gene is known as a common hepatogenic marker. RT-PCR analysis of albumin gene expression showed that UCB-derived MSCs could express albumin gene in a time-dependent manner in the differentiating medium containing FGF-4 and HGF. The analysis revealed progressive increase in albumin expression from day 14 till day 28 of culture. No albumin expression was observed in the 7th day sample and the control specimen (Fig. 5).

4. Discussion

The characterization of stem cells present in adult BM and UCB has exposed that UCB-derived MSCs are similar to bone marrow-derived MSCs with respect to cellular properties and multi-lineage differentiation potential.^{17–19} However, UCB-derived MSCs have a low risk of immunoreactivity and can be isolated without any harm of the baby and no ethical concerns.⁸

MSCs should be isolated from UCB sample immediately or within 6 h of collection²⁰ because the activity and the quantity of MSCs in HUCB are low and decrease with time. In this study, MSCs were isolated from 3 out of 22 samples, which was in accordance with the previous study done by Kang et al. who isolated MSCs from 10 out of 39 samples.¹²

In the present study, isolated MSCs from UCB were heterogeneous during the first few days. Cells adhering to the bottom of culture flasks were observed on day 4–5 and were fibroblast-

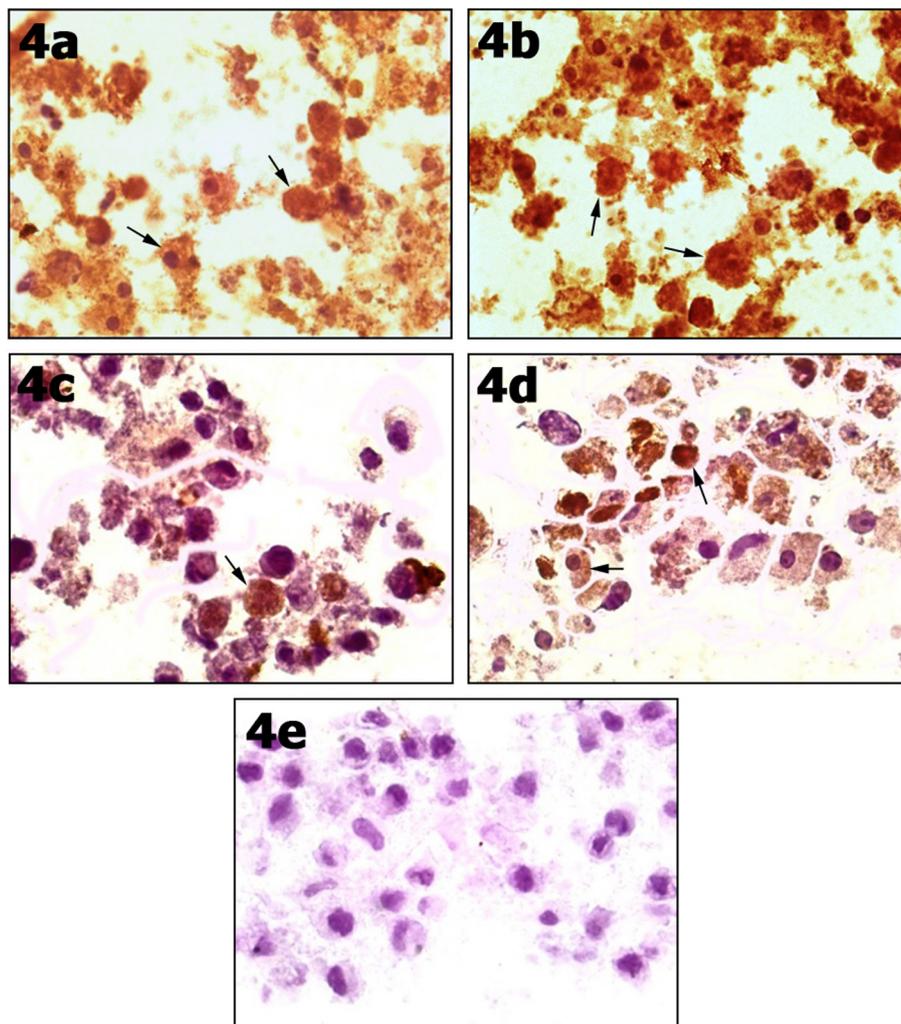


Figure 4 After 7 days of culturing UCB-MSC under hepatogenic conditions by treatment with FGF-4 and HGF, cells showed positive cytoplasmic reaction for AFP (arrows) (4a). At day 14 under hepatogenic conditions, almost all cells showed positively stained cytoplasm (arrows) (4b) and some cells were still positive by day 21 (4c) and day 28 (4d). Control negative cells cultured in serum free media showed undifferentiated cells stained negative for AFP (4e). Immunocytochemistry stain for AFP $\times 100$.

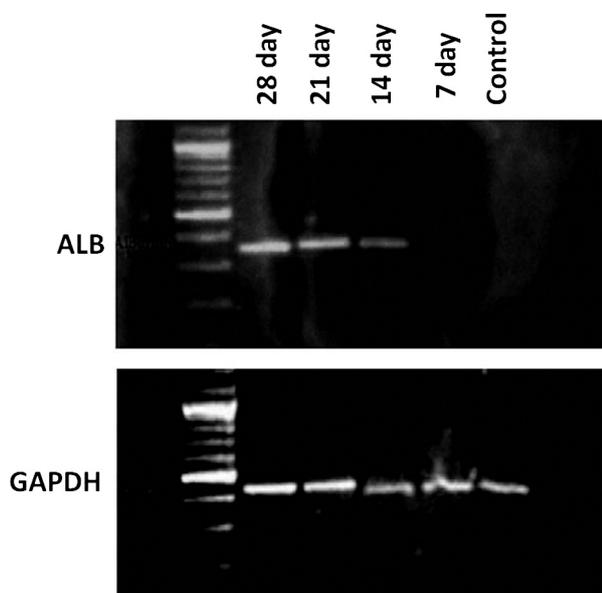


Figure 5 A photograph showing reverse transcription polymerase chain reaction (RT-PCR) assays for albumin mRNA which was expressed by hepatocyte-like cells derived from MSC. The bands indicating the expression of albumin appeared at days 14, 21 and 28. No expression was detected at day 7 of differentiation, and the control sample. GAPDH mRNA, which was used as a control gene, was expressed in the 5 specimens (7, 14, 21, 28 days) under hepatogenic conditions and the control specimen.

like. The sub-cultured cells were much purer and fibroblast-like, with disappearance of other cells. According to set of standards proposed by The International Society for Cellular Therapy, a cell can be classified as a MSC when having plastic adherent properties under normal culture conditions and a fibroblast-like morphology. In fact, some claim that MSCs and fibroblasts are identical functionally.²¹ In the present study the primary culture cells were allowed to grow to 70% confluence with a density of MSCs 1×10^6 , prior to induction of differentiation by growth factors. Schwartz et al.⁵, working on bone marrow derived progenitor cells, reported that a higher cell density favored hepatogenesis, whereas densities less than 12.5×10^3 failed to give rise to hepatocyte-like cells.

In the previous studies, differentiation of stem cells derived from UCB or matrix into hepatocytes was induced through addition of different mixtures of cytokines and growth factors like fibro-blast growth factor (FGF), hepatocyte growth factor (HGF), oncostatin M, epidermal growth factor, leukemia inhibitory factor, insulin-transferrin-selenium premix and others.^{22,10,23–26}

Hepatocyte growth factor (HGF) was first recognized as a blood-derived mitogen for hepatocytes. HGF and its receptor c-Met are the key factors for liver growth and function²⁷ and in helping the proliferation of hepatocytes²⁸ and liver regeneration.²⁷ HGF stimulates the proliferation of hepatic progenitors to repair hepatic injury.²⁹ Adult human bone marrow MSCs were induced to differentiate into hepatocytes *in vitro* after being cultured with only HGF.³⁰

Fibroblast growth factor-4 (FGF-4) is mitogenic for fibroblasts and endothelial cells. Mouse embryonic stem cells could differentiate into cells expressing hepatocyte-specific genes and

antigens when FGF-4 was added to the culture medium.³¹ However, Schwartz et al. determined that the two essential growth factors for proper hepatocyte differentiation are FGF-4 and HGF.⁵

This study showed that with the induction of HGF and FGF-4 the UCB-MSCs could expand *in vitro* and differentiate into hepatocyte-like cells. By day 7, some cells started to differentiate by acquiring a cuboidal or round form. At day 14, the number of differentiated cells increased and expressed albumin gene as detected by RT-PCR analysis. However, the secretion of the protein was significantly detected only by the 28th day. Urea secretion was significantly increased at the 21st day and elevated further by the 28th day. This indicates that MSCs started to differentiate as early as the 7th day but started to function by the 21st day of culture under hepatogenic conditions. These results are closely similar to those of Kang et al. who used the same hepatogenic induction technique by HGF and FGF-4.¹² However, they noticed earlier secretion of albumin (by day 16) detected by radioimmunoassay. They found also progressive increase in AFP level till day 28. In this study the AFP immuno-positive cells became less toward the 28th day. A similar observation has been found in hepatocytes derived from BM progenitor cells.⁵ AFP is considered an early developmental marker of hepatoblasts formation. So, the decrease of its expression by some cells with time may indicate more maturation of hepatocyte-like cells.

5. Conclusion

This study demonstrated that UCB-derived MSCs had the ability to differentiate into hepatocyte-like cells starting from the 7th day after culturing under hepatogenic conditions and became well functioning at days 21 and 28. These data indicated that UCB-derived MSCs can be a promising source of cell therapy for intractable liver diseases.

Conflict of interest

We have no conflict of interest to declare.

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