African Journal of Biotechnology Vol. 10(5), pp. 840-847, 31 January, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1104
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Mesenchymal stem cells differentiate into hepatocyte-like cells under different induction systems in hepatitis B patients with liver failure

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Accepted 23 December, 2010

This study aimed to investigate and compare induced differentiation of mesenchymal stem cells from hepatitis B (HB) patients with liver failure into hepatocytes-like cells with different induction systems *in vitro*. The differentiation of MSCs from HB patients with liver failure was induced *in vitro* into hepatocytes-like cells by three cell culture media (serum-free medium (group 1), auto serum-containing medium (group 2) and medium supplemented with fetal bovine serum (FBS) (group 3)). Cell morphology, cell growth curve, amount of urea and glycogen and mRNA expressions of ALB, CK18 and AFP were detected and compared. Morphological changes in group 1 and 2 were more evident than that in group 3 and cell growth in group 3 was faster than the other two groups. The amount of urea and glycogen in group 1 and 2 was significantly elevated when compared with that in group 3 after 6 days of culture. RT-PCR analysis indicated the mRNA expressions of ALB, CK18 and AFP in group 1 and 2 were markedly increased as compared to that in group 3. The differentiation of MSCs from HB patients with liver failure into hepatocytes-like cells can be induced by three different cell culture media and the inductive effects were more profound in cells grown in the serum-free medium and auto serum-containing medium.

Key words: Liver failure, mesenchymal stem cells, hepatogenic differentiation, *in vitro.*

INTRODUCTION

Marrow mesenchymal stem cells (MSCs) are characterized by self-renewal and multi-directional differentiation (Pei, 2003; Jiang et al., 2002; Brazelton et al., 2000). MSCs have the following advantages, easy isolation and cultivation, expansion potential, stable phenotype, compatibility by different delivery methods and minor side effects after implantation (Bayes-Genis et al., 2002; Mackenzie and Flake, 2001; Fang et al., 2004; Le Blanc and cell therapy and tissue engineering.

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Abbreviations: MSCs, Marrow mesenchymal stem cells; **HGF,** hepatocyte growth factor; **FGF-4,** fibroblast growth factor 4; **EGF,** epidermal growth factor; **HB,** hepatitis B; **FBS,** fetal bovine serum; **AFP,** α-feto protein; **ALB,** albumin; **CK-18,** cytokeratin-18; **RT-PCR,** reverse transcriptase- polymerase chain reaction.

Pittenger, 2005; Gao et al., 2007; Yao et al., 2005; Lee et al., 2004). Studies have confirmed they played important roles in the Autogenetic MSCs do not cause immunological rejection and have been clinically used in France, Germany, Japan, Italy and China (Gao et al., 2007; Yao et al., 2005; Lee et al., 2004). We have applied autogenetic MSCs in the treatment of hepatitis B (HB) patients with liver failure and satisfactory therapeutic effects were achieved (Gao et al., 2007). To improve the therapeutic effectiveness of autoplastic transplantation and investigate the mechanism of MSCs differentiation, biological characteristics of MSCs from HB patients were explored in our previous studies (Peng et al., 2007a; Peng et al., 2007b; Zheng et al., 2008; Xie et al., 2009) and found MSCs from HB patients were more difficult to cultivate *in vitro*, grown more slowly with worse activity, faster aging process and less passage generations, than MSCs from normal human.

On the base of our previous studies, the present study

aimed to investigate the characteristics of differentiation of MSCs from HB patients with liver failure into hepatocytes-like cells. Serum-free medium and auto serum-containing medium were applied to avoid the side effects of foreign proteins and cells maintained in the medium supplemented with fetal bovine serum (FBS) were used as the controls. We tried to establish a safe and effective hepatogenic derivation system *in vitro* of MSCs from HB patients with liver failure.

MATERIALS AND METHODS

Ethical considerations

The present study was approved by the local Ethical Committee of the 3rd Affiliated Hospital of Sun Yat-Sen University and informed consent was obtained before study.

Collection of bone marrow and MSCs culture

A total of 5 ml of bone marrow was aspirated from anterosuperior iliac spine and mixed with 5 ml of PBS, followed by centrifugation at 1500 r/min for 5 min. The supernatant was removed and cells were suspended in 5 ml of PBS. Then, cell suspension was added to 5 ml of Percoll separating medium (Sigma Company, USA), followed by centrifugation at 2500 r/min for 30 min. Nucleated cells in the middle layer were obtained and rinsed with 5 ml of PBS, followed by centrifugation at 1500 r/min for 5 min. The supernatant was removed and cells were suspended in L-DMEM supplemented with 10% FBS. The cells were grown in a 25 cm^2 culture flasks and incubated at 37°C in an atmosphere containing 5% CO_2. The culture medium was changed after three days and then every two days. MSCs were digested with 0.25% Trypsin and 0.1% EDTA and passaged (1:2) when 70 to 80% cell fusion occurred.

Preparation of autoserum from HB patients with liver failure

Autoserum from HB patients with liver failure was prepared through storage at 4°C for 30 min, followed by centrifugation at 2000 r/min for 30 min. Then, the autoserum was sterilized by filtration with 22 µm filter, followed by inactivation at 56°C for 30 min and stored at -20°C.

Flow cytometric analysis

The third passage of MSCs from HB patients with liver failure were digested, rinsed with PBS and grown at a density of 1.0 × 106 cells/ml. The cells were incubated with FITC-CD44 antibody, PerCP-CD45 antibody and PE-CD34 antibody (BD Biosciences Company, USA) and detected with flow cytometry (FACScan, BD Biosciences, USA) using mouse isotype IgG1 as the control. Amplifier mode was linearity mode, flow rate was low, signals and threshold were set and the gate was set at the target cells.

Hepatic differentiation of MSCs *in vitro*

The third passage of MSCs from HB patients with liver failure was plated at a density of 5 × 10^5 cells/cm^2 in 25 cm^2 cell culture flasks. The cells were grown in three different media which were replaced every 3 days. Group 1 (Serum-free medium), HepatoZYME-SFM (Gibco, USA) supplemented with 20 ng/ml HGF, 20 ng/ml FGF-4 and 20 ng/ml EGF (PeproTech, USA); Group 2 (auto serum-containing medium), L-DMEM (Gibco, USA) supplemented with 10% (v/v) autoserum, 20 ng/ml HGF, 20 ng/ml FGF-4 and 20 ng/ml EGF; Group 3 (medium supplemented with FBS), L-DMEM (Gibco, USA) supple- mented with 10% (v/v) FBS (Gibco, USA), 20 ng/ml HGF, 20 ng/ml FGF-4 and 20 ng/ml EGF.

Cell morphology

After 7, 14 and 21 days of culture, the morphology of MSCs was observed under an inverted phase contrast microscope (M20-35DX, Olympus Company, Japan) and their differences were compared, respectively.

Cell growth curve

The third passage of MSCs were seeded at an initial concentration of 1000 cells/cm^2 in three 24-well plates and independently maintained in different media. Cells were detached with 0.25% Trypsin- 0.02%EDTA and the number of cells was counted with a hemocytometer from day 1 to 12 Dead cells were excluded by Trypan blue staining (Sigma-Aldrich, USA). All of these experiments were performed in triplicates for each time point and cell growth curve was drawn.

Detection of urea production

The concentration of urea in the supernatant was colometrically measured according to the manufacturer's instructions (Urea assay kit, Sigma-Aldrich, USA) after 24 h treatment with 6 mM NH_4Cl (Sigma-Aldrich, USA) at different time points (day 0, 6, 9, 12, 15 and 18).

Detection of glycogen production

The concentration of glycogen in the supernatant was analyzed by periodic acid-Schiff (PAS) staining at different time points (day 0, 18 and 21). Cells that were adherent to the slide were fixed with 95% alcohol for 15 min and then rinsed thrice with ddH_2O. Subsequently, after oxidization in 1% periodic acid for 15 min, slides were treated with Schiff's reagent for 30 min, followed by washing with ddH_2O. Samples were then incubated with Mayer's hematoxylin for 2 min and observed under a phase contrast microscope.

Total RNA isolation and quantitative RT-PCR

Total RNA was extracted from MSCs at different time points (day 6, 9, 12, 15 and 18) using RNAprep cell kit (Tiangen Biotech Beijing, China) according to the manufacturer's protocol. Complementary DNA was synthesized with 2 µg of total RNA using reverse transcriptase (TaKaRa, Japan) with oligo-dT primer (TaKaRa, Japan) in a 25 µl reaction mixture. Primers used for amplification were listed in the Table 1. The expression level of β-actin was used as an internal control to normalize specific gene expression in each sample. Real-time quantitative RT-PCR using the ABI Prism 5700 Sequence Detection System was performed with 2 µl of the single-stranded cDNA with SYBR Green PCR master mix (Applied Biosystems, USA). The DNA was denatured at 93°C for 2 min, followed by 40 cycles of amplification. Each cycle consisted of denaturation at 93°C for 30 s, annealing at 55°C for 40 s and extension at 60°C for 1 min, with an additional extension at 60°C for 7 min. Each sample was measured in duplicates. The mRNA expressions of ALB, CK18 and AFP were analyzed and compared.

Table 1. Primers used for amplification (sequences were from Genebank, primers and probes were designed by Primer express 2.0).

Gene	Forward primers	Reverse primers	Probes
H-ALB	5'-TCTTACCAAAGTCCACACGGAAT-3'	5'-GGTCCGCCCTGTCATCAG-3'	5'-AM-CTGCCATGGAGATCTGCTTGAAT-TAMRA-3'
H-CK18	5'-TGGCGAGGACTTTAATCTTGGT-3'	5'-TGGTCTTTTGGATGGTTTGCA-3'	5'- FAM-TGCCTTGGACAGCAGCAACTCC-TAMRA -3'
H-AFP	5'-GGAGCGGCTGACATTATTATCG-3'	5'-TGGCCAACACCAGGGTTTA-3'	5'-AM-CACTTATGTATCAGACATGAAATGACTCCA-TAMRA-3'
H-β-actin	5'-GCGCGGCTACAGCTTCA-3'	5'-TCTCCTTAATGTCACGCACGAT-3'	5'- FAM-CACCACGGCCGAGCGGGA-TAMRA -3'

The expression of different genes was normalized by β-actin.

Statistical analysis

SPSS 13.0 statistical software was used for analysis and Kruskal- Wallis Test was performed. A value of $P < 0.05$ was consi dered statistically significant.

RESULTS

Flow cytometry analysis

Flow cytometry analysis showed that MSCs (the third passage) from HB patients with liver failure were positive for CD44 and negative for CD34 and CD45 which was consistent with that in normal adults (Figure 1).

Morphological changes of MSCs

Morphological changes were observed in MSCs of all groups, which began at 7 days of culture, finished successfully at 14^{th} and 21^{st} day after induction, respectively in 3 groups, which were from fusiform shape to polygon and similar to spherical. MSCs in group 1 looked like spreading out and aging at 21^{st} day, while these similar aging morphologic changes were not found in groups 2 and 3 (Figure 2).

Cell growth curves

The growth of cells in group 1 was the lowest and it was the highest in group 3, but without any statistical significance ($P = 0.257$) (Figure 3).

Urea assay

Urea was detected in the supernatant of three groups at different time points and the production of urea was in a time-dependent manner. Additionally, the concentration of urea in groups 1 and 2 was significantly higher than that in group 3 after 6 days of culture ($P = 0.025$ and 0.031, respectively) (Figure 4).

Glycogen assay

No glycogen was measured at the beginning of culture and, at 18 and 21 days of culture, glycogen assay was observed positively in all groups. Morphological changes and increased amount of glycogen in group 1 and 2 were found to be more evident and poor inductive effects were obser- ved in culture medium supplemented with FBS (Figure 5).

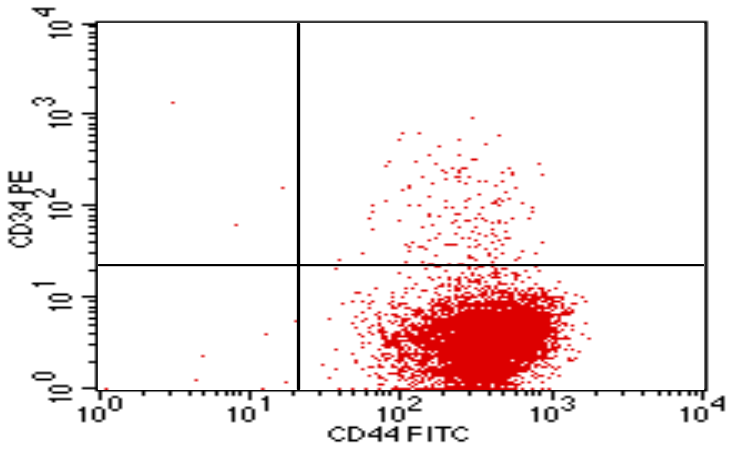
Real time quantitative RT-PCR analysis

With prolonged culture period, the mRNA expression of ALB and CK18 were gradually increased and that of AFP was decreased in all groups, indicated by quantitative mRNA analysis. Changing tendency of ALB mRNA curve in group 1 showed the most significant inclination among the 3 groups ($P = 0.025$) (Figure 6), while there were not significant difference in inclinations of CK18 mRNA and AFP mRNA curve among 3 groups ($P = 0.085$ and 0.595, respectively) (Figure 6).

DISCUSSION

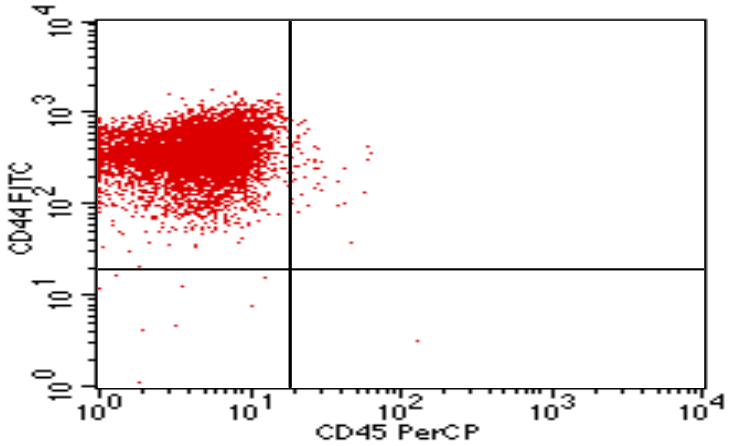
The induction of differentiation potential of MSCs has been confirmed by numerous studies (Lee et al., 2004; Fu et al., 2002; Tippi et al., 2001), however, the differentiation potential of MSCs from HB patients with liver failure was poorly understood. The present study was to explore the differentiation of MSCs from HB patients with liver failure into hepatocyte-like cells on the basis of the biological characteristics of MSCs (Peng et al., 2007a). In the present study, cell morphology was observed and secreted functional proteins were measured. Furthermore, the inductive effects of three representative differentiation culture media were com- pared. The results indicated serum free medium and auto serum containing medium exerted better inductive effects on the differentiation of MSCs into hepatocyte-like cells.

After induction with different media, MSCs became polygon or oval and the expressions of



File: CD44/CD34/CD45.003
Acquisition Date: 04-Sep-06
Gated Events: 8241
Total Events: 11910

Quad	Events	% Gated
UL	3	0.04
UR	169	2.05
LL	7	0.08
LR	8062	97.83



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Acquisition Date: 04-Sep-06
Gated Events: 8241
Total Events: 11910

Quad	Events	% Gated
UL	8190	99.38
UR	42	0.51
LL	8	0.10
LR	1	0.01

Figure 1. Expression of surface molecule CD34, CD44, CD45 of MSCs (3rd passage) of HB patients with liver failure.

albumin (ALB), cytokeratin-18 (CK-18) and α-feto protein (AFP) were detected to different extents. ALB and CK18, which are the markers of mature hepatocytes, were lowly or almost not expressed in hepatic progenitor cells. However, AFP was secreted by hepatic progenitor cells and could not be detected as cells become mature. However, with prolonged period of induction, the expression of ALB and CK18 was gradually increased and that of AFP was not detected until 5 days of culture. The expression of α-feto protein (AFP) was decreased with prolonged culture period. These findings were consistent with other studies (Zhou et al., 2004; Avital et al., 2001); the results suggested that, differentiation of MSCs from HB patients with liver failure could be induced into hepatocyte-like cells *in vitro*.

The results of this study also showed that, serum-free medium and auto serum-containing medium were better than medium supplemented with fetal bovine serum (FBS) in the ability to induce differentiation. Furthermore, the greatest advantages of these two media are that immunological rejection and heterologous virus infections are avoided. HepatoZYME-SFM which is one of the serum-free media consists of a variety of trace elements and can exert promotive effects on the survival and proliferation of primary hepatocytes and on the differentiation of bone marrow cells. It can induce the clonal differentiation of bone marrow cells into hepatocyte-like cells and accelerate these cells to express differentiation markers after stimulation with hepatocyte growth factor (HGF), fibroblast growth factor 4 (FGF-4) and epidermal growth factor (EGF) (Miyazaki et al., 2002; Zhou et al., 2004). But at the late stage, cellular senescence was observed and the number of cells dropped, which may be related with deficiency in nutrients under serum free conditions.

A variety of studies has been conducted to explore the differentiation of MSCs into hepatocyte-like cells induced by serum containing culture medium in which the serum was separated from animals with liver failure (Zhou et al., 2004; Yamazaki et al., 2003). It is well known that, when liver is injured, endogenous HGF will promote the transformation of stem cells and induce them into hepatocytes exerting repair effects. Our study confirmed that auto serum-containing medium, which contained growth factors such as HGF, FGF-4 and EGF, could confer better

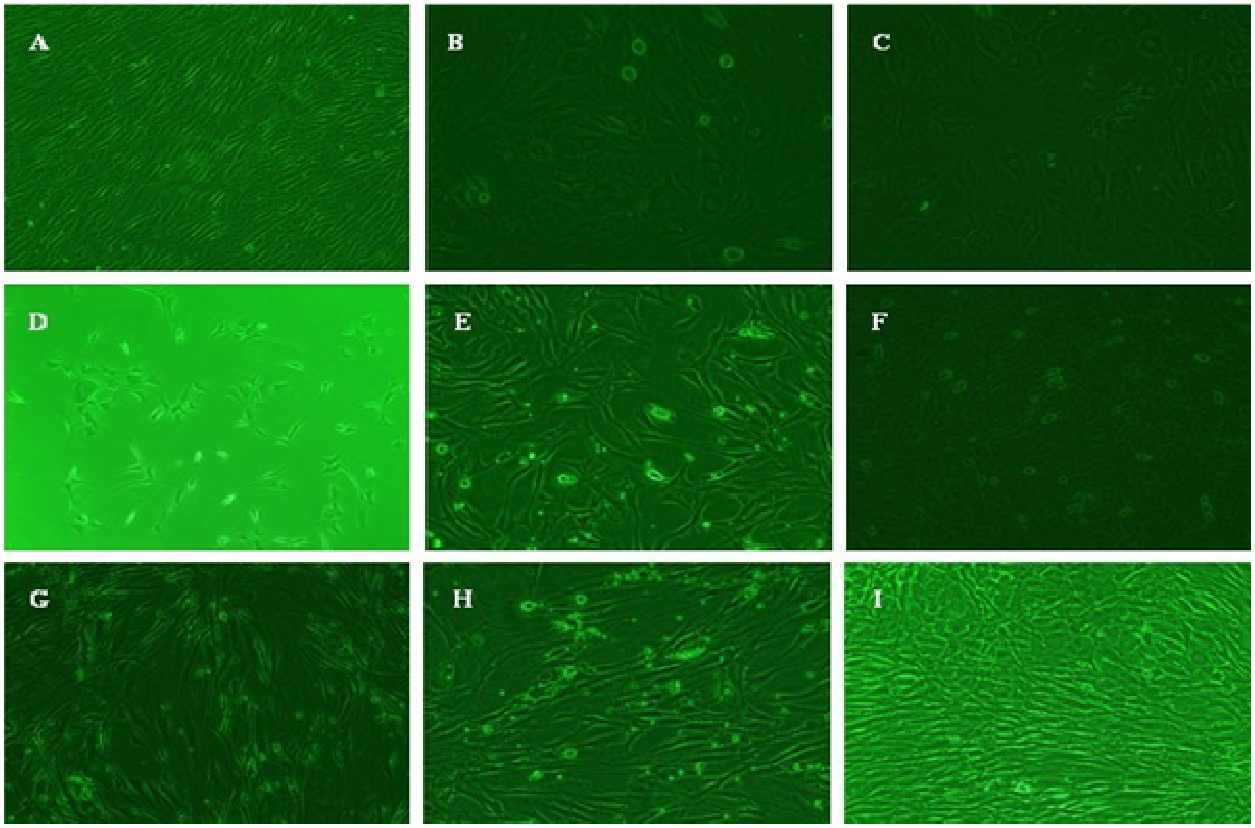

Figure 2. Morphology of MSCs in the induction process (100×). Cells in groups 1, (A-C: d7, d14 and d21); 2, (D-F: d7, d14 and d21); 3, (G-I: d7, d14, d21).

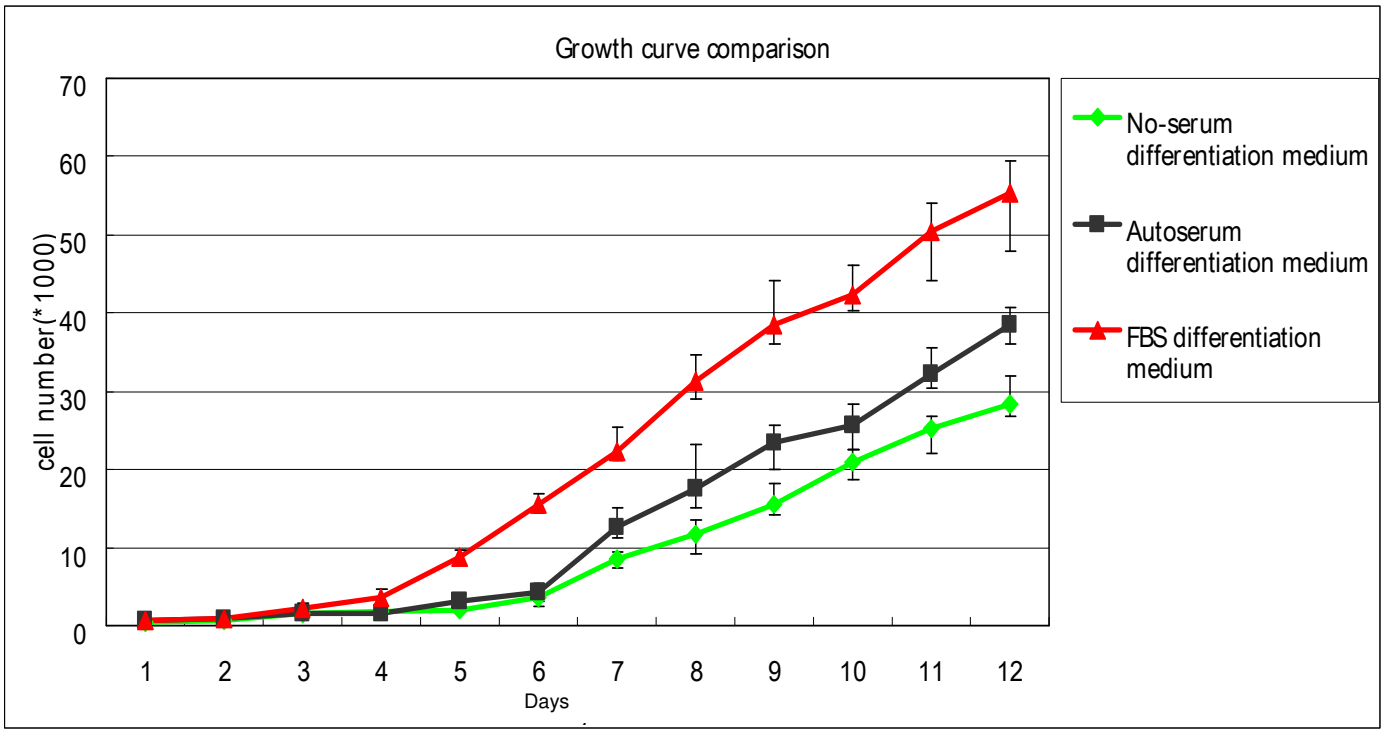

Figure 3. Growth curve of MSCs in the induction process.

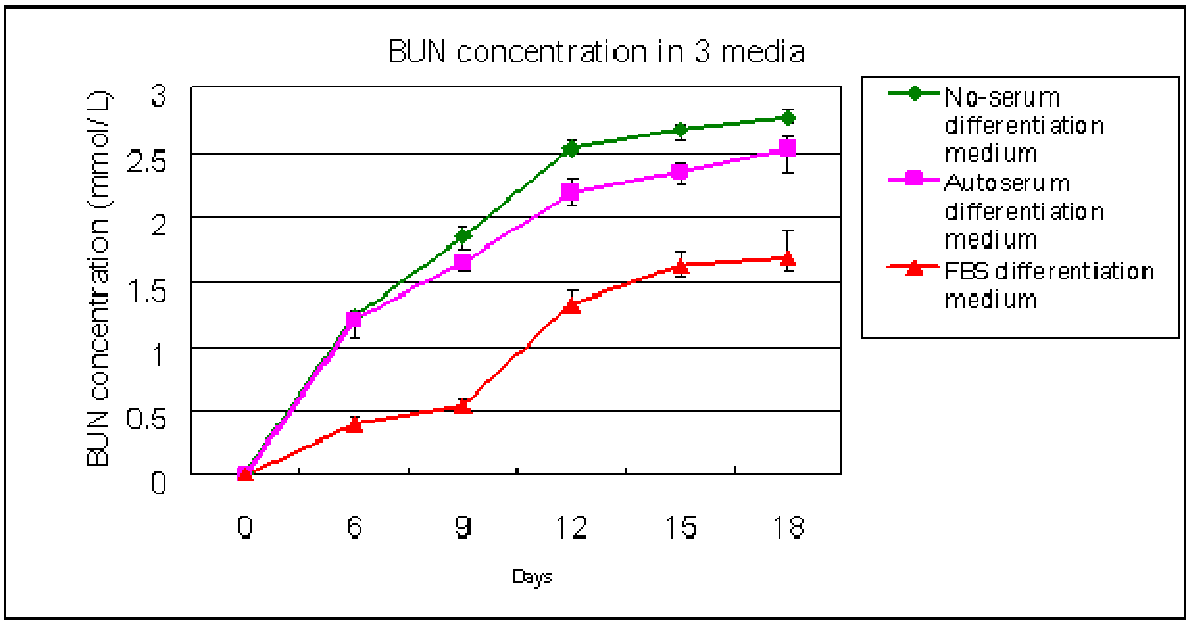

Figure 4. BUN concentration comparison in the induction process.

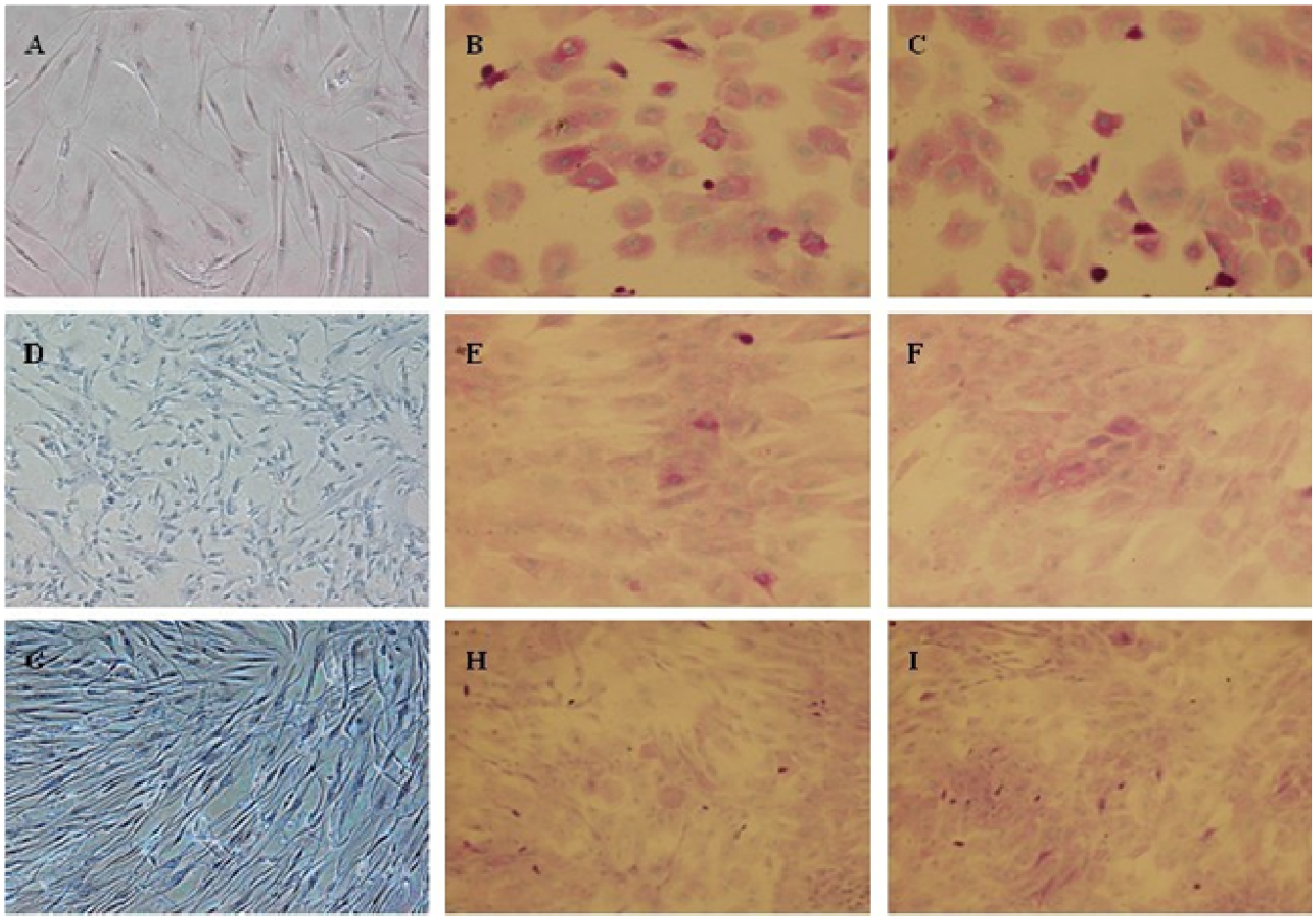

Figure 5. Glycogen production assays of MSCs in the induction process (100×). Cells in group 1, (A-C: d0, d18 and d21); 2, (D-F: d0, d18 and d21); 3, (G-J: d0, d18 and d21).

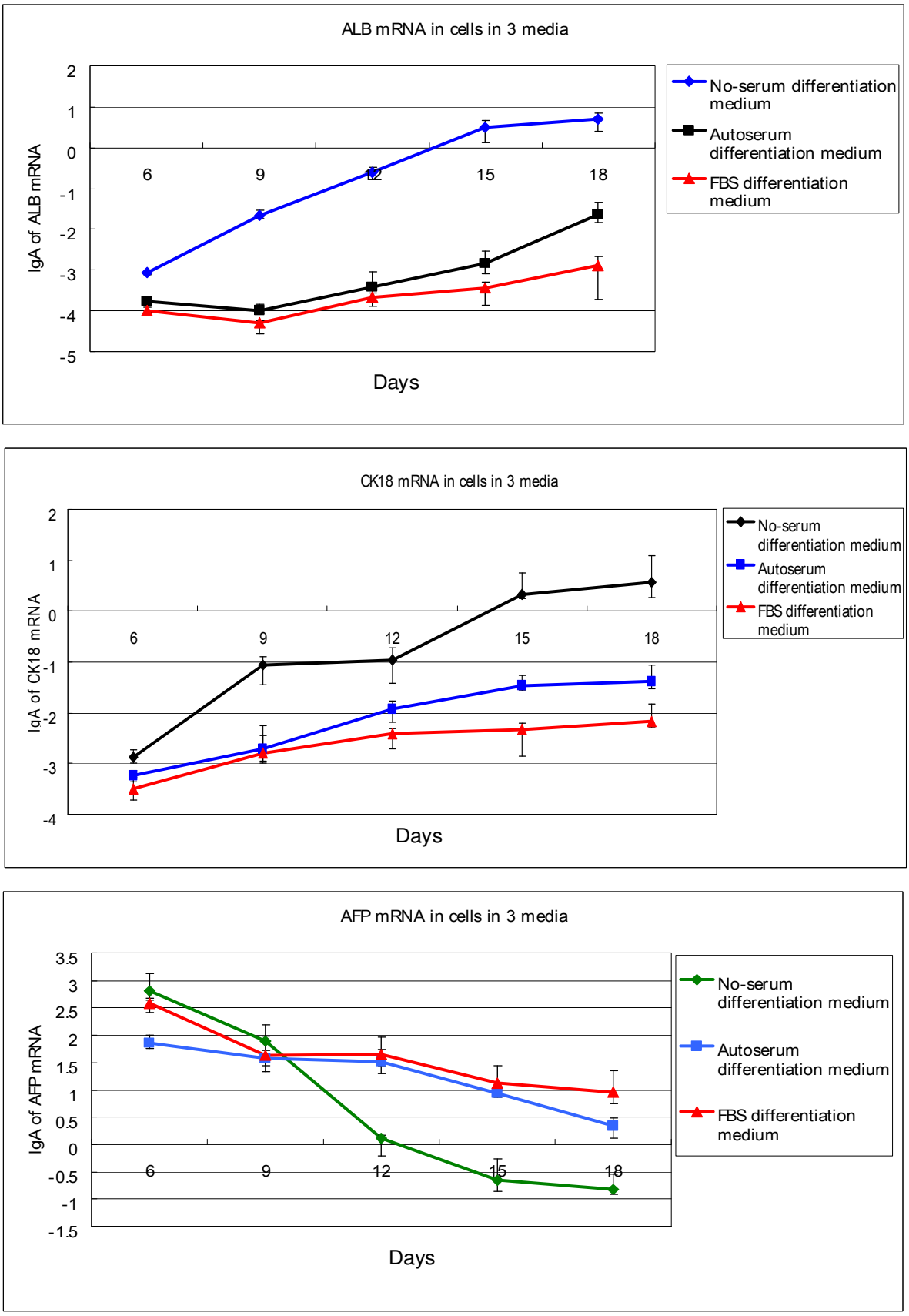

Figure 6. Quantitation assays of ALB mRNA, CK18 mRNA and AFP mRNA of MSCs in the induction process.

induction effects on the differentiation of MSCs and these findings were consistent with previous studies (Miyazaki et al., 2002; Oh et al., 2000).

Therefore, we postulated that autoserum containing medium may be a favorable candidate in the *in vitro* inductive differentiation of MSCs from patients with liver failure. Furthermore, the autoserum is collected from patients, so it is easy to obtain and accompany it by better biosafety. In addition, growth of induced cells is stable and cellular senescence is seldom observed in autoserum containing medium. Because autoallergic MSCs transplantation has potential advantages in avoiding immunological rejection and tumor genesis, the specific mechanisms and clinical application of MSCs have been the focuses in the researches. It was shown that, not every patients can get satisfactory effect in both domestic and international researches (including ours), so we believe that the small number of transplanted cells, the micro environmental disruption of inductive differentiation *in vivo* and the collapse of the structure of hepatic lobule are all important restricting reasons. Although it's not determined that, MSCs or hepatocyte-like cells after inductive differen- tiation is better for transplantation *in vivo*, for the reasons mentioned earlier, it is necessary to explore a safe and effective way to induce differentiation of MSCs *in vitro* for patient with liver failure. This study provides a research foundation for further exploration of therapeutic application of stem cells in liver failure. Our study also provides a basis for the clinical application of stem cells in HB patients with liver failure.

ACKNOWLEDGEMENTS

This study was supported by the Natural Science Fund of Guangdong Province (No. 9451008901001973), New Teacher Fund of Ministry of Education (No. 20090171120079), Medical Technology Project Funds of Dongguan City (No.200910515022), and National Undeca-Five Project Fund, China (No. 2008ZX10002-005).

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