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Full Length Research Paper

Correlation between morphological and biological characteristics of mesenchymal stem cells and hepatocytes derived from rat mesenchymal stem cells *in vitro*

Mohammad Hadi Bahadori[1,2]*, Bahram Soltani[1], Abtin Heidarzadeh[3], Fatemeh Ghasemian[4] and Shervin Ghadarjani[1]

[1]Cellular and Molecular Research Center, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran.
[2]Department of Anatomy, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran.
[3]Department of Community Medicine, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran.
[4]Department of Biology, Faculty of Biology, Kharazmi (Tarbiat Moallem) University, Tehran, Iran.

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Hepatocyte dysfunction with the possibility of eventual organ failure is created from most liver diseases. Images of cell morphology can be obtained nondestructively using a conventional inverted microscope. Therefore, this study attempted to investigate several morphological parameters of mesenchymal stem cells (MSCs) and MSCs-derived hepatocytes, *in vitro*, and their correlation with biological activities. Our results show that MSCs are clonogenic and self-renewal and these cells showed apoptotic property. There were significant difference in confluence rate and doubling time rate among serial passages (P<0.01). When MSCs were cultured with fibroblast growth factor – 4 (FGF-4) and hepatocyte growth factor (HGF), approximately 80-90% of cells became small, round and epithelioid on day 21 that was increasingly similar to hepatocytes in appearance. Compared with the control, levels of alpha-fetoprotein (AFP), albumin (ALB) and urea increased significantly from day 12, 16 and 20, respectively and were higher on day 24 (P < 0.01). The AFP, ALB and urea production level of the large polygonal cells was markedly higher than that of cells with other morphologies. In conclusion, morphological parameters such as polygonal index, cell adhesion area, morphologic changes, proliferation and double nucleoli rate might be use as an indication of differentiation of bone marrow-MSCs into hepatocytes and their functions.

Key words: Hepatocyte, stem cell, morphological parameter, polygonal index.

INTRODUCTION

Hepatocyte dysfunction with the possibility of eventual organ failure is created from most liver diseases. Hepatocyte transplantation as an alternative to whole liver transplantation for treatment of hepatocyte disease is still hampered by the limited availability of marginal donor organs to isolate human adult hepatocytes, insufficient amount and quality for transplantation (Stock et al., 2008). The availability of donor organs is limited and many of these patients die each year waiting for liver transplantation (Shi et al., 2005). Moreover, the most suitable kind of stem cells for therapy of liver is bone marrow mesenchymal stem cells (BM-MSCs) (Xie et al., 2009; Yokoyama et al., 2008) and nowadays the usage of *in vitro* hepatocyte models may be the only suitable way for drug metabolism and hepatotoxicity studies (Kulkarni and Khanna, 2006; Brandon et al., 2003). BM-MSCs are useful both the clinical application and basic science perspectives (Aurich et al., 2007; Xie et al., 2009), hence, they can be an ideal resource for the

*Corresponding author. E-mail: bahadori@gums.ac.ir. Tel: +98-131-6690884/ 6690099. Fax: +98-131-6690007.

functional hepatocytes (Shi et al., 2005). The differentiation rate *in vitro*, has been measured by the quantitative real time – polymerase chain reaction (RT-PCR) determination of albumin (ALB), α-fetoprotein (AFP), cytokeratin 19 (CK19) (Shi et al., 2005; Ling et al., 2008; Koenig et al., 2006) and cytokeratin 18 (CK18) (Shi et al., 2005; Shi et al., 2008; Koenig et al., 2006; Abdel et al., 2007) mRNA expression level, which are some of the hepatic-specific genes. An analysis of the cell surface antigens CD29 and CD44 may be used to distinguish whether the MSCs differentiation into hepatocytes is happening (Shi et al., 2008; Ling et al., 2008). Unfortunately, due to limitation of cells removed from patients and destructive properties of these measurements, this method is not applicable to cells used for autologous transplantation (Takagi et al., 2008). However, images of cell morphology can be obtained nondestructively using a conventional inverted microscope (Takagi et al., 2008).

Takagi et al. (2008) reported that the function of cells is correlated with their morphological properties. Herein, we have investigated some kinds of morphological properties of BM-MSCs and hepatocytes derived from BM-MSCs, which is one of the most important morphological characteristics of hepatocyte cells during differentiation of MSCs is the polygonal index. However, there has been no specific report regarding morphological characteristics of hepatocytes during differentiation of MSCs.

Consequently, because as developing a nondestructive monitoring method for usage of MSCs and their differentiation into hepatocytes, cell morphological characteristics and biological activity during differentiation culture were assayed.

MATERIALS AND METHODS

Sprague-Dawley rats (4 to 6 weeks old) were purchased from Razi Institute (Iran). The rats were kept with a 12 h light/dark cycle and were fed free food and water. All animals were treated in accordance with the guidelines of the Guilan University of Medical Science (GUMS) University Ethics Community Standards on the Care and Use of Laboratory Animals.

Materials and reagents

Medium DMEM, Trypsin–EDTA and FBS were purchased from Gibco (UK), PBS from Merck Pharma (Germany), bFGF and HGF were purchased from R&D systems (USA), TUNEL solution from Roche (Germany) and dexamethasone, indomethacine, ascorbic 2-phosphate, β glycerophosphate, Triton X-100, sodium citrate, Insulin, PI, methylisobutylxanthine, hematoxylin, periodic acid, Schiff's reagent, penicillin and streptomycin were from Sigma–Aldrich.

Bone marrow mesenchymal stem cells

For isolation of rat MSCs; female Sprague-Dawley rats (weighing 200 to 250 g) were killed by intraperitoneal administration of a lethal dose of sodium pentobarbital. The femurs and tibias were carefully dissected away from attached soft tissue as previously reported with modification (Lei et al., 2007). The ends of the bones were cut and the bone marrow was aseptically extruded with 5 ml PBS solution by using a syringe with a 21G needle and flushing the shaft ten times. The marrow tissue was dissociated by pipetting. The cell suspension was then centrifuged at 500 × g for 5 min and the supernatant was discarded. Bone marrow mesenchymal stem cells were then mechanically dispersed into a single-cell suspension so that the density of BMSCs reached 1×10^6 cells/ml. At this point, marrow cells were plated in a 25 cm² plastic flask in DMEM containing 20% FBS, 100 U/ml penicillin, and 100 mg/ ml streptomycin. All cells were incubated at 37°C, in an atmosphere of 5% humidified CO_2. After 48 h incubation, the non-adherent cell populations were removed and the medium was added and replaced every three or four days for about two weeks. When the cells grew to 80% confluency, they were harvested with 0.25% trypsin and 1 mM EDTA for 5 min at 37°C, replated and diluted 1:3 on a 25 cm² plastic flask, after which it was again cultured to the next confluency and harvested.

The whole experiment was replicated for the marrow of three rats; statistical analysis was performed using the SPSS 13.0 statistical package (SPSS). All outcomes were assessed using Chi-squared test and a level of 0.05 or less was considered significant. At the end, the 4th passage cells from either group were differentiated towards hepatocyte lineages as well as bone and adipose cells (Ke et al., 2008).

Measuring the MSCs dimensions and colony-forming assay

For measuring the MSCs dimensions, the length and width (the broadest part of the cells) of the fibroblastic MSCs from confluenced culture were measured using the objective micrometer mounted on the inverted microscope. After 14 days, colonies that were formed from single cells were observed by light microscopy, counted and the mount of colonies per dish was determined. The measurement of cells was determined by counting more than 600 cells in multiple fields. Each measurement was replicated in three separate experiments.

Cell proliferation assay

In order to examine proliferative ability of isolated cells from BM with different cell densities, plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and cells were serially passaged for more than 24 days. DT, the time taken for the number of cells to duplicate, is an important index of cell growth rate in culture. In this study, DT was calculated for cultures during passages 1 to 4 using the equation for DT (culture duration / PDN). PDNs were calculated for each passage using the equation $PDN = \log N / N_0 \times 3.31$, where N is the number of cells at the end of culture period and the N_0 is the cell number at culture initiation (Eslaminejad et al., 2008).

TUNEL assay

To examine apoptosis by TUNEL assay, apoptotic cells were detected as previously reported with modification (Yadav et al., 2010). In brief, first, the 4th passage cells cultivated on cover slips were washed twice with PBS (0.2% BSA) at 4°C. After fixation and washing, cells were incubated in TUNEL solution for 60 min at 37°C in dark environment. After washing of cells with PBS (twice), they were stained with PI for live nuclear localization. Samples were analyzed using fluorescence microscope (IX71, Olympus, Japan), with the range of 570 to 620 nm and PI with the range of 330 to 380

nm by measuring number of green cells ($TUNEL^+$) and PI (binding to DNA, red cells).

Differentiation culture conditions

Adipogenic and osteogenic differentiation of confluenced 4th passage cells was induced by culturing the cells in DMEM containing related inducer agents (Kang et al., 2006). One week after induction, adipogenic differentiation was assessed by the cellular accumulation of neutral lipid vacuoles that were stained with oil-red O and after 14 days, osteogenic differentiation was evaluated by alizarin red staining (Eslaminejad et al., 2008) and observed with light microscope. When the cells grew to 80% confluence, the control group was continuously cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin (growth medium: GM).

The hepatocyte differentiation group was cultured in DMEM supplemented with 10% FBS, 20 ng/ml HGF, 10 ng/ml FGF-4, 100 U/ml penicillin and 100 U/ml streptomycin (differentiation medium: DM) and medium changed every 3 days. After 21 days, hepatogenic differentiation was evaluated by PAS staining (Kang et al., 2006).

Analysis of cell morphology

A CCD camera (DP71, Olympus, Japan) and a microscope (IX71, Olympus, Japan) recorded the cultured cell images. Adhesion area in one cell was measured using Photoshop (Adobe, San Jose, CA). In order to detect polygonal morphology from endothelial-like elongated morphology, largest diameter of the cell was measured as the major axis and polygonal index was defined using the Equation;

$$\text{Polygonal index} = \frac{\text{Cell adhesion area}}{(\text{Major cell axis})^2}$$

It has been reported that cells that had both an adhesion area larger than 4000 μM^2 and a polygonal index larger than 0.3 were considered large polygonal cells (Takagi et al., 2008).

Alpha-fetoprotein and Albumin assay

Values of AFP and ALB in the changed medium were measured by analyzing the culture medium. An ELISA was performed on days 0, 3, 6, 9, 12, 15, 18, 21 and 24 and was measured using a polyclonal antibody to rat albumin (a quantitative ELISA-based test kit, Innovative Research, USA) and rat alpha-fetoprotein (ELISA kit, Uscn Life Science inc. China).

Urea assay

The concentration of urea in culture media was measured using a colorimetric assay (Quantichrom Urea assay kit, Bioassay Systems, Brussels, Belgium) according to the manufacturer's instructions. Samples from 5 separate cultures were analyzed in triplicate for each condition.

Long-term culture of BM-MSCs for evaluation of spontaneous differentiation

Sub-confluent cultures of MSCs (~ 3×10^5 cells/25 cm^2 plastic flask) were maintained in growth medium (GM). The culture medium was changed every 3 days, and cells were continuously cultured for 3 months. Morphology and characterization of MSCs noticed and the percentage of each cell type under inverted microscope following specific staining for evaluation of spontaneous differentiation were evaluated.

RESULTS

Cultured bone marrow cells indicated morphological characteristics of MSCs

The morphology of adherent cells gave the evidence to support the idea that culture-expanded cells from BM might be MSCs. As the culture progressed with trypsinization, round and non-adhesive cells gradually decreased and eventually disappeared by week 1. Fibroblast-like cells remained homogeneous morphologically. These cells showed prolonged proliferative capacity without any morphological changes for more than 6 passages (over 3 months) and had a differentiation potential to mesenchymal derivatives including osteoblasts and adipocytes. A confluence rate average of 7 days for 1st passage, 8 days for 2nd passage, 9.5 for 3rd passage and 8 for 4th passage were detected in which there was significant difference in confluence rate of passages during this culture period ($P< 0.01$).

According to our data, the length and width of MSCs from primarily adherent cultures appeared to be 23.18 ± 4 and 6.5 ± 0.6 µM, respectively. After 14 days, colonies derived from single cells were visible by light microscopy. Differences in rate of cell proliferation caused formation of colonies with different size and cell density. Average colony-forming efficiency of BM-MSCs was determined as 18.28 ± 6.06 for BM. Results are shown as mean ± standard deviation of 3 plates in each group. There were no significant differences. Also, the growth rate of the mesenchymal cells in serial passages was different. In this regard our results exhibited DT for the passages 1 (DT= 1.84 days), 2 (DT = 2.16 days), 3 (DT= 2.44 days) and 4 (DT= 2.42 days). There was significant difference in DT of 3rd and 4th passage cells compared with 1st and 2nd passages ($P< 0.01$). In addition, measurement of cell doubling time in our study was 2.21 days. More also, observations from the TUNEL staining indicated that a number of TUNEL-positive cells were found at the untreated MSCs (2.98%). However, low rate of apoptotic cells was distinguished with usage of TUNEL.

Mesenchymal stem cells shape changed into small, round and epithelioid in the presence of induction by FGF-4 and HGF

When cells reached 80% confluence, they were treated with hepatocyte differentiation medium. Up to the 8-day treatment, they did not show any change in comparison to the control. After treatment, cell remained quiescent

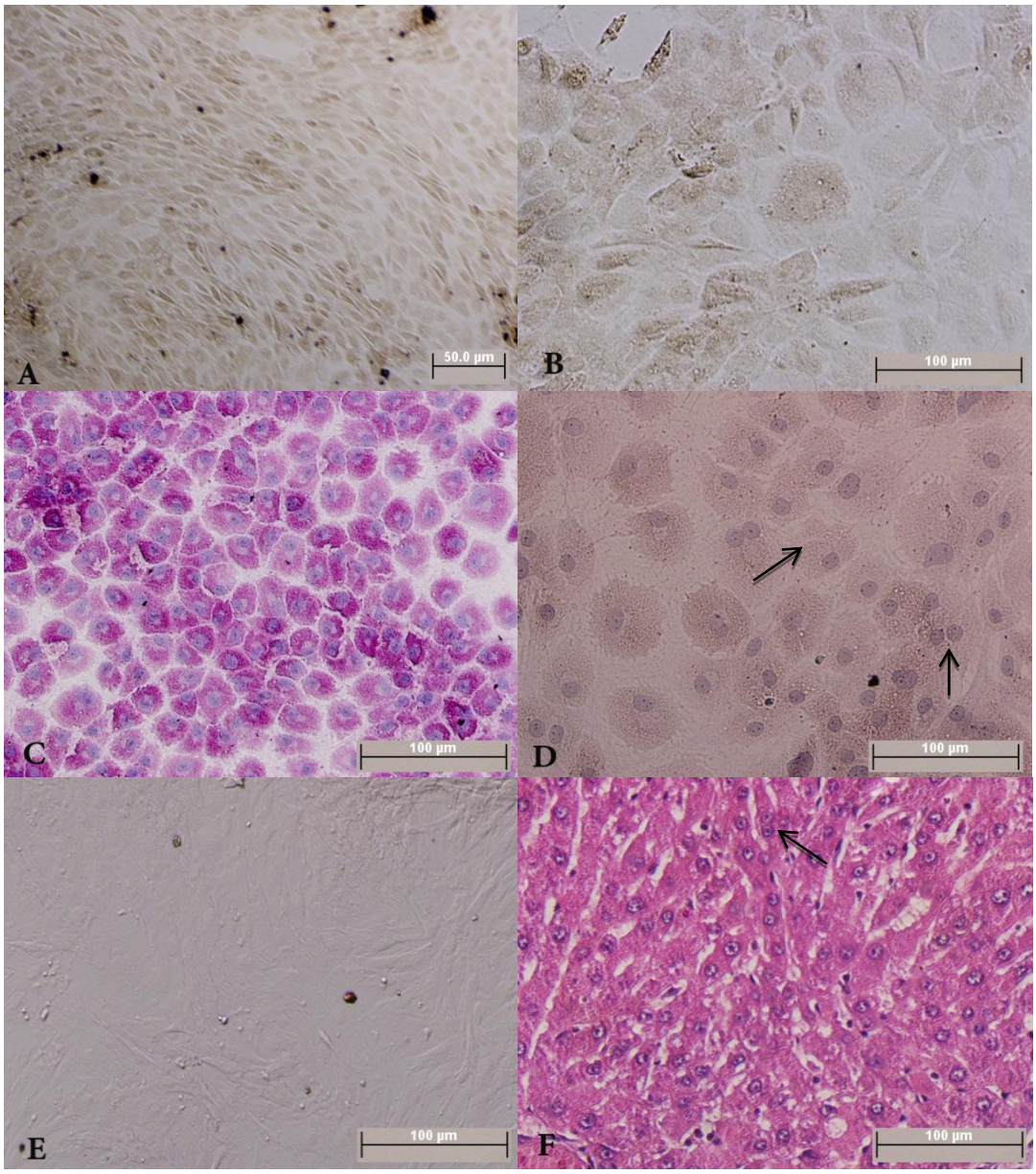

Figure 1. Changes in cell morphology and glycogen storage potential of BM-MSCs during hepatic differentiation. BM-MSCs cultured with optimized hepatocyte differentiation medium: (a) for 0 days (Undifferentiated cells assumed bipolar fibroblast-like morphology and a fraction of the cells gradually turned into small round cells during differentiation transition stage); (b) for 9 days; (c) for 21 days (Most differentiated MSCs turned into oval cells during the maturation stage and the rate of MSCs morphological change was 80 to 90%), upregulated glycogen storage was shown by means of PAS staining and cuboidal morphology typical of hepatocytes is seen; (d) Hematoxylin staining of differentiating hepatocytes. Hematoxylin staining revealed granular cytoplasm. The picture also shows bi-nucleated cells, indicative of mature hepatocyte-like cells (arrow). (E) MSCs did not show Glycogen storage, after staining with PAS on 20 days. (F) Double nucleoli cells in in-vivo liver tissue (arrow).

and small, round cells appeared in the treatment group, as well as epithelioid cells. On day 21, approximately 80 to 90% of cells were small, round or epithelioid that was increasingly similar to hepatocytes in appearance. The control cells were still fibroblast-like, though cell proliferation continued and overlapped in some regions.

During the initiation step, MSCs were observed as bipolar fibroblast-like morphology (Figure 1). We speculate that the induced cells possessed the morphologic features of hepatocytes. Under hepatogenic conditions, the fibroblastic morphology of MSCs gradually progressed toward the polygonal morphology of hepatocytes in a

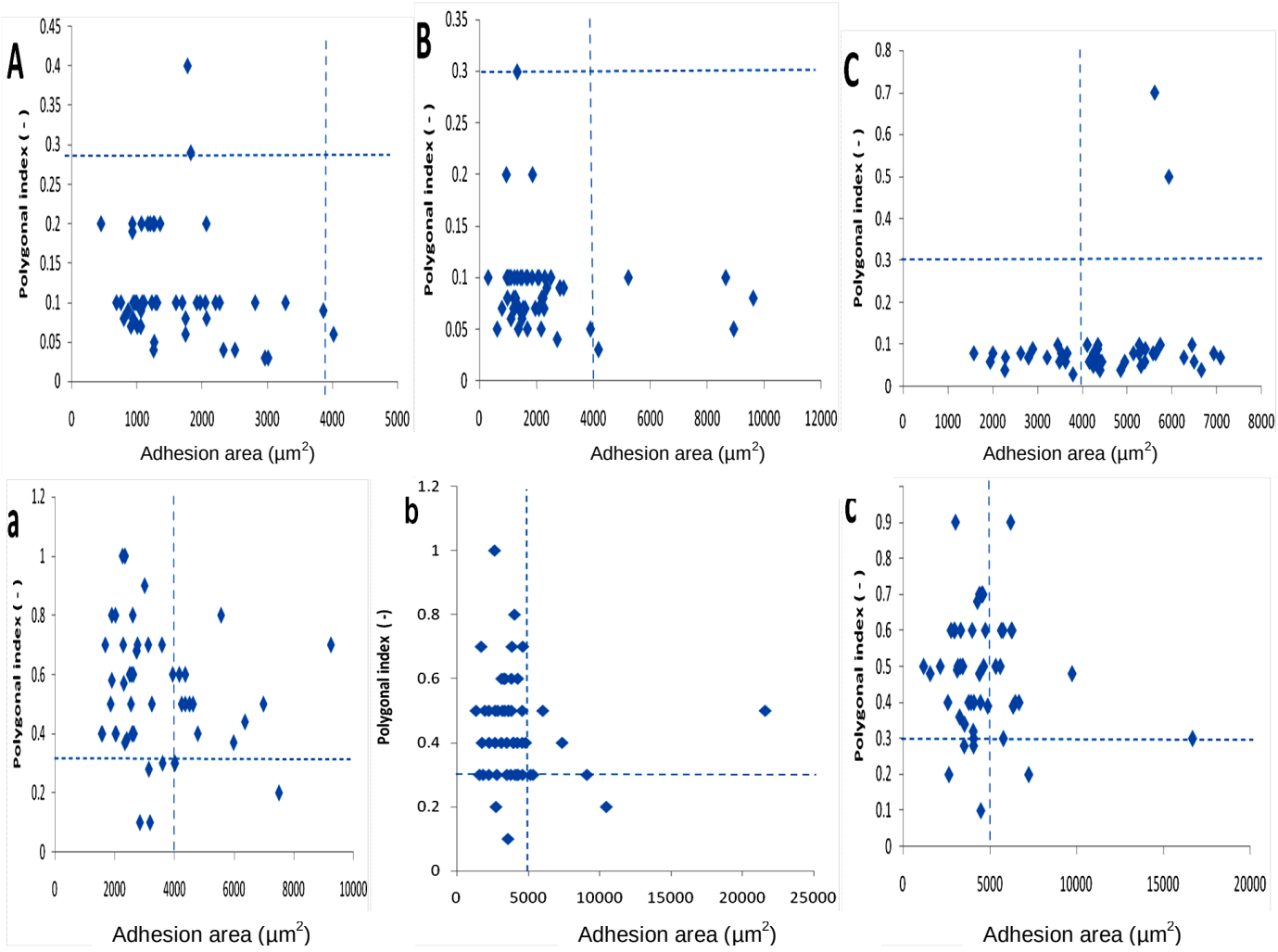

Figure 2. Analysis of cell morphology during differentiation culture. Cell polygonal index was plotted against cell adhesion area for individual cells observed at 9 (A and a), 15 (B and b) and 24 days (C and c) during culture with an inoculum density of 1.5 × 10^5 cells/cm^2 using DM (a, b and c) or GM (A, B and C).

time-dependent manner and became apparent by 1 week post-induction. However, the mature cuboidal morphology with granulated structures was not fully developed until 8 days post-induction. Our results show that the percentage of bi-nucleoli is 17.1% in the induced group with growth factors compared with double nucleoli rate *in vivo* liver tissue (14%; Figure 1F) did not show significant differences.

Analysis of cell morphology and biology activities during differentiation culture

Mesenchymal stem cells were cultivated for 24 days with a cell density of 1 × 10^5 cells/cm^2 using GM or DM. Cells grew in both cultures using the two types of media. At first, a typical fibroblast-like cell shape was observed at 24 h in both cultures, which was maintained thereafter in the culture using GM. Then, polygonal cells increased in number in the culture with DM. Cell polygonal index was plotted against cell adhesion area for cells (n > 50) in images taken at 9, 15 and 24 days of the cultures (Figure 2). Cells having both a large adhesion area and a large polygonal index increased in number during the culture using DM (13, 15 and 52%, respectively). Adhesion area increased as the time progressed, but polygonal index almost remained without change, whereas large polygonal cells were rarely found in the culture using GM that increased in number in the culture using DM. There was significant difference in number of large polygonal cells in 24th day of culture in DM in comparison to other days and when compared with cultured cells in GM, too. We could successfully maintain these cultures up to 24 days in the optimized hepatocyte differentiation medium.

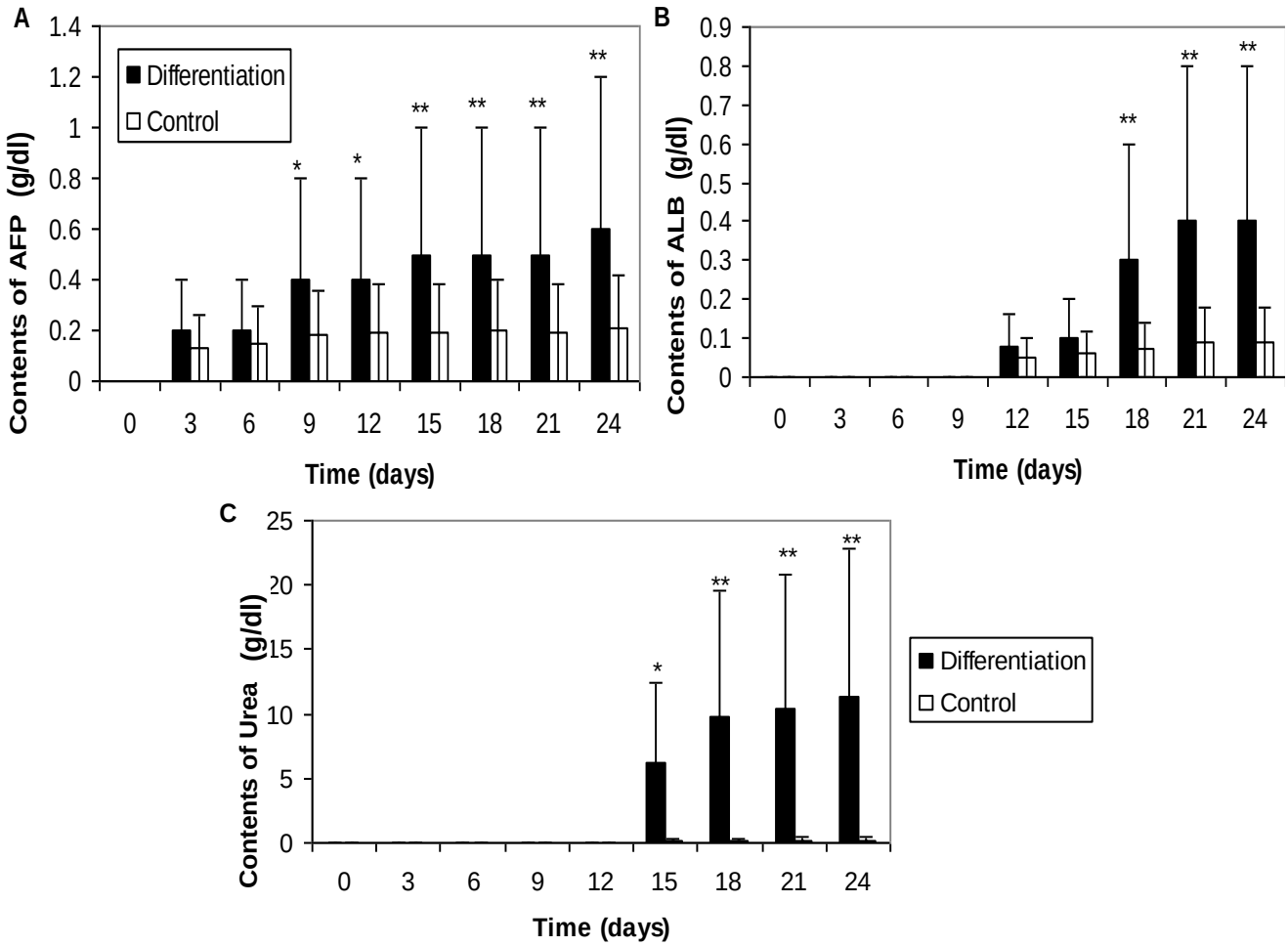

Figure 3. Contents of AFP, albumin and urea in the medium at different time points. (A) The levels of AFP increased significantly from day 9 in MSCs cultured with FGF-4 and HGF, continued and was higher on day 24. (B) The level of albumin could not be detected by ELISA before day 12, albumin increased significantly on day 18 and continued to day 24. (C) Urea produced by MSCs was first detected on day 15, and increased to 2/ 2 ± 4/11 g/dl on day 24. * $P < 0.05$ vs control, ** $P < 0.01$ vs control.

This suggests that these hepatocyte-like cells differentiated from MSCs will prove to be an important tool and a better alternative to be for used in animals for hepatotoxicity studies.

By ELISA, albumin and AFP production was measured at various time points throughout differentiation. Compared with the controls, the levels of AFP increased significantly from day 9 with a concentration of 0.4 ± 0.1 g/dl ($P < 0.05$) in MSCs cultured with FGF-4 and HGF, continued to increase and was higher on day 24 ($P < 0.01$) with a concentration of 0.6 ± 0.2 g/dl. The level of albumin could not be detected by ELISA before day 12, albumin increased significantly on day 18 with a concentration of 0.3 ± 0.1 g/dl ($P < 0.01$), and still to 0.4 ± 0.1 g/dl on day 24 ($P < 0.01$).

Urea production and secretion by hepatocytes were detected at various time points throughout differentiation. By treatment with FGF-4 and HGF, urea produced by MSCs was detected with a concentration of 9.8 ± 2.5 g/dl on day 18, and increased to 11.4 ± 2.2 g/dl on day 24 ($P < 0.01$, Figure 3). Each measurement was replicated in three separate experiments. In addition, a spontaneous differentiation assay of BM-MSCs into neurons and adipocytes was performed that elucidated whether BM-MSCs could spontaneously differentiate into mature neurons and adipocytes or not.

The MSCs were cultured on plates in the medium without any extra growth factor. After a total of 2 to 3 months' cultivation, cells were observed for morphological analysis (Figure 4A to C). Results showed that during the spontaneous differentiation process, about 5 to 6% cells possessed the neuronal morphological characteristics. The results indicate that BM-MSCs have the potential to differentiate into nerve-like cells spontaneously. Also, our results showed that after 4 weeks of MSCs culture, a few MSCs (1 to 2%) exhibited cellular accumulation of neutral lipid vacuoles that were stained with oil-red O (Figure 4D).

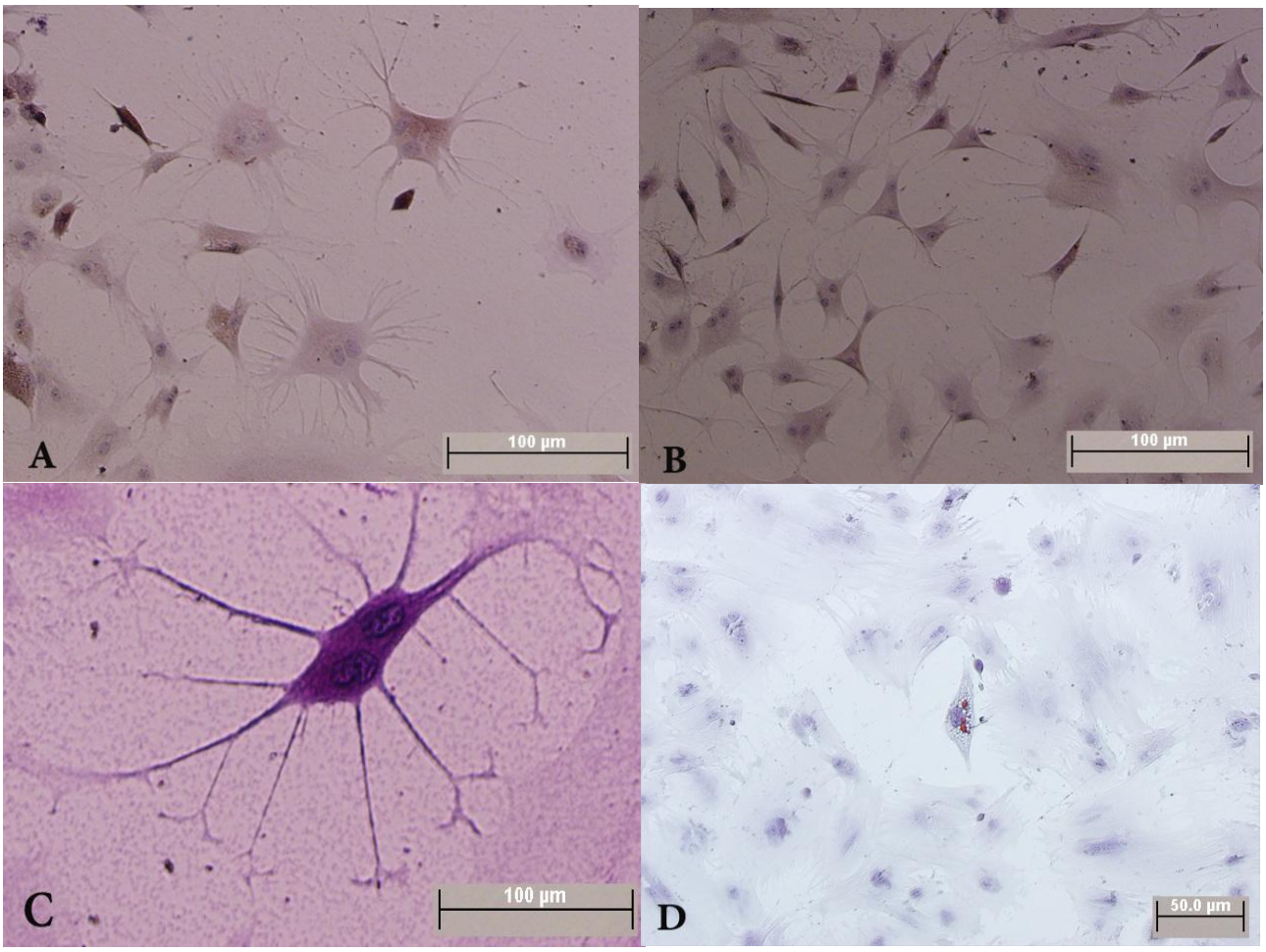

Figure 4. Morphological changes of spontaneous differentiation of MSCs into nerve-like cells and adipogenesis potential**.** (A) A flat MS cells assumed neuronal morphological characteristics progressively (on 13 days), (B) then intersected as a network structure. (C) Exhibiting a typical neuronal appearance (on 23 days). (D) MSCs showed differentiation potential to adipocytes spontaneously (oil-red O and hematoxylin staining).

DISCUSSION

A large number of fresh marrow cells might be necessary for clinical approaches; in particular, it can be very difficult to get enough cells for effective therapy from patients with severe hepatic disease (Kang et al., 2006). Our results showed that proliferative rate is different among passage-1 cells compared with late passages and there was significant difference among them. This study was designed to investigate the most morphological characteristics of MSCs and MSC-derived hepatocyte. As the culture progressed with trypsinization, round and spindle-shaped cells gradually decreased and eventually disappeared by Week 1. Fibroblast-like cells however remained homogeneous morphologically. These cells showed prolonged proliferative capacity without any morphological changes for more than 6 passages (over 3 months) and had a differentiation potential to mesenchymal derivatives including osteoblasts and adipocytes (unpublished data). These characteristics were similar with the results from BM-MSCs reported by Lee et al. (2004)Our data indicated that the percentage of apoptosis in MSCs is 2.98%, which is similar to data obtained by Yadav et al. (2001) whose results reported that apoptosis rate in MSCs is 2.3%.

In addition, measurement of cell doubling time in our study was 53.23 h, which is similar to doubling time (50.57 h) reported by Eslaminejad et al. (2008). Cells of isolated and passaged in our study showed stable phenotype after serial passaging. Furthermore, Kang et al. (2006) indicated that FGF-4 and HGF induced MSCs into cells with morphological and functional characteristics of hepatocytes. The results of this study suggested that it is not important to culture MSCs on whatever materials *in vitro*. Li et al. (2010) introduced a three-dimensional (3D) bioscaffold for differentiation of rat BMSCs into hepatocytes. In their study, differentiated hepatocyte-like cells in C-PLGA (collagen-coated poly

[lactic-co-glycolic-acid]) scaffolds expressed hepatocyte-specific markers at mRNA and protein levels. However, the mature cuboidal morphology with granulated structures is not fully developed until 8 days post-induction. We obtained a high proportion (80 to 90%) of a homogeneous population of cells, with morphological features of hepatocytes. These cells exhibited oval shape morphology with polygonal structure and were bi-nucleated. The percentage of large polygonal cells was initially very low (13%, at 9 days) and increased to 52% (at 24 days) during the culture in DM. These results suggest that the cells differentiated into hepatocytes in differentiation medium.

Our results are also similar to that of Yokoyama et al. (2008) that reported that the percentage of large polygonal cells increased during differentiation of MSCs into chondrocytes lineage. Therefore, FGF-4 and HGF induced MSCs into cells with morphological and functional characteristics of hepatocytes. In our study, the cells that were differentiated into hepatocyte-like cells could produce urea, secrete albumin, AFP and store glycogens. Urea production was characterized by hepatocyte activity, although kidney tubular epithelium also produced urea. In contrast, albumin and AFP production tested for the presence and metabolic activity of hepatocytes. Hepatocytes can generate and store glycogens. In our research, we found that AFP could be detected throughout the differentiation due to a low level of AFP in the medium. From day 9, the levels of AFP increased significantly compared with the controls, suggesting that MSCs began to secrete AFP. Before day 12, the concentration of albumin could not be measured by ELISA. Levels of albumin and urea production and secretion by hepatocytes were increased on day 24 as also reported by Kang et al. (2006).

In addition, the results of this study are in agreement with the findings of Lam et al. (2010), which indicated that the elevation of albumin and AFP expression in MSCs undergoing hepatic differentiation is time-dependent. Our results show that the AFP, ALB and urea production level of the large polygonal cells was markedly higher than that of cells with other morphologies. Therefore, this study indicate potential of MS-cells differentiation into hepatocyte under induced conditions with FGF-4 and HGF, in which the BM-MSCs differentiated into hepatocytes could be characterized using morphological parameters such as polygonal index, cell adhesion area, morphological change and double nucleoli appearance. Hence, cell morphological characteristics and biological activity during differentiation culture can be a nondestructive monitoring method for usage of MSCs and their differentiation into hepatocytes.

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Abbreviations: BM-MSCs, Bone marrow-mesenchymal stem cells; **MSC,** mesenchymal stem cell; **bFGF,** basic fibroblast growth factor; **HGF,** hepatocyte growth factor; **DMEM,** Dulbecco's modified eagle's medium; **FBS,** fetal bovine serum; **PI,** propidium iodide; **PBS,** phosphate-buffered saline; **DT,** doubling time; **PDN,** population doubling number; **GM,** growth medium; **DM,** differentiation medium; **PAS,** periodic acid-schiff; **TUNEL,** terminal transferase dUTP nick end labeling; **ELISA,** enzyme-linked immunosorbent assay; **AFP,** alpha-fetoprotein; **ALB,** albumin; **CK,** cytokeratin.

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