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• *BASIC RESEARCH* •

Fibroblast growth factor-4 and hepatocyte growth factor induce differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocytes

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Abstract

AIM: To investigate the differentiation of human umbilical cord blood (HUCB)-derived mesenchymal stem cells (MSCs) into hepatocytes by induction of fibroblast growth factor-4 (FGF-4) and hepatocyte growth factor (HGF), and to find a new source of cell types for therapies of hepatic diseases.

METHODS: MSCs were isolated by combining gradient density centrifugation with plastic adherence. When HUCB-derived MSCs reached 70% confluence, they were cultured in Iscove modified Dulbecco medium (IMDM) supplemented with 10 mL/L FBS, 20 ng/mL HGF and 10 ng/mL FGF-4. The medium was changed every 4 d and stored for albumin, alpha-fetoprotein (AFP) and urea assay. Expression of CK-18 was detected by immunocytochemistry. Glycogen storage in hepatocytes was determined by PAS staining.

RESULTS: By combining gradient density centrifugation with plastic adherence, we could isolate MSCs from 25.6% of human umbilical cord blood. When MSCs were cultured with FGF-4 and HGF, approximately 63.6% of cells became small, round and epithelioid on d 28 by morphology. Compared with the control, the level of AFP increased significantly from d 12 to 18.20±1.16 μg/L (t = 2.884, P<0.05) in MSCs cultured with FGF-4 and HGF, and was higher (54.28±3.11 μg/L) on d 28 (t = 13.493, P<0.01). Albumin increased significantly on d 16 (t = 6.68, P<0.01) to 1.02±0.15 μg/mL, and to 3.63±0.30 μg/mL on d 28 (t = 11.748, P<0.01). Urea (4.72±1.03 μmol/L) was detected on d 20 (t = 4.272, P<0.01), and continued to increase to 10.28±1.06 μmol/L on d 28 (t = 9.276, P<0.01). Cells expressed CK-18 on d 16. Glycogen storage was observed on d 24.

CONCLUSION: HUCB-derived MSCs can differentiate into hepatocytes by induction of FGF-4 and HGF. HUCB-derived MSCs are a new source of cell types for cell transplantation therapy of hepatic diseases.

Key words: Mesenchymal stem cell; Differentiation; Hepatocyte

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INTRODUCTION

Cell transplantation therapy is an alternative to the treatment of liver conditions that sustain significant liver injury. It has been reported that bone marrow-derived MSCs can differentiate into mesoderm cells such as osteoblasts[1-3], adipocytes[4-6], neural and brain cells[7-10],cardiomyocytes[11-13] and hepatocytes[14,15] *in vivo* or *in vitro*. However, it has been demonstrated that the number and the differentiating potential of bone marrow MSCs decrease with age [16]. Therefore, search for alternative sources of MSCs is of significant value.

Studies have shown that human umbilical cord blood (HUCB) contains hematopoietic stem cells and MSCs, both of them can be used as alternative sources to bone marrow for cell transplantation therapy. Hematopoietic stem cells of HUCB have already been proven to be useful for the treatment of various hematological disorders. On the other hand, identification of the MSCs remained elusive until they were recently isolated as a homogeneous cell population by a number of different laboratories[17,18]. The extensive characterization of these cells has revealed

that they are similar to bone marrow-derived MSCs not only with respect to cellular properties and multi-lineage differentiation potential, but also with respect to their molecular context[19,20]. It was reported that HUCB-derived MSCs could differentiate into mesoderm cells such as osteoblasts, adipocytes[21], neuron-like cells[19, 22], and cardiomyocytes[23, 24]. Schwartz *et al*[14]. and Kang *et al*[15]. reported that FGF-4 and HGF could induce differentiation of bone marrow MSCs into functional hepatocyte-like cells. We hypothesized that FGF-4 and HGF could also induce differentiation of HUCB-derived MSCs into functional hepatocyte-like cells.

Using a relatively simple method, namely gradient density centrifugation in combination with adherence, we isolated HUCB-derived MSCs and cultured them in plastic culture flasks by induction of FGF-4 and HGF.The results showed that HUCB-derived MSCs could differentiate into hepatocytes *in vitro*.

MATERIALS AND METHODS

Materials and reagents

Human cord blood was obtained from Department of Obstetrics and Gynecology, First Hospital of Xi' an Jiaotong University. Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Material Co.Ltd. Albumin RIA kit and alpha-fetoprotein RIA kit were purchased from Beijing Atom High-tech Co.Ltd. Mouse anti-human monoclonal antibody cytokeratin 18(CK-18) was purchased from Chemicon Company. Iscove modified Dulbecco medium (IMDM) was purchased from GIBCO. Acid fibroblast growth factor-4 (FGF-4) and hepatocyte growth factor (HGF) were purchased from R&D Systems, Inc. Ficoll-Paque (1.077 g/mL) was pursed from Amersham-Pharmacia.

Isolation and culture of MSCs from HUCB

Ten mL of umbilical cord blood was taken from each patient (n = 49).The blood sample was diluted with an equal volume of D-Hanks. The diluted umbilical cord blood was layered over an equal volume of Ficoll-Paque (1.077g/mL). Mononuclear cells (MNCs) were recovered from the gradient interface and washed twice with D-Hanks after centrifugation at 800r/min for 20 min. The MNCs were suspended in 5 mL IMDM supplemented with 100 mL/L FBS, 100 U/mL penicillin, 100 U/mL streptomycin, at last plated in 25 cm^2 plastic cell culture flasks at the density of 10^6 /mL. The cells were maintained at 37 ℃ in 50 mL/L CO_2 in fully humidified air. The medium was changed for the first time on d 5 and then changed every 4 d. Because of limited time, 27 blood samples were treated within 3 h, 22 samples within 3-6 h, 8 samples within 6-12 h, and 2 samples after 12 h.

Hepatocyte differentiation

The cultured cells were harvested from the culture bottles with 0.25g/L trypsin. Cultured cells at passage 3 were seeded in six- well cell culture clusters. When the cells grew at 70% confluence, the control group was continuously cultured in IMDM supplemented with 10 mL/L FBS, 100 U/mL penicillin, 100 U/mL streptomycin. The hepatocyte differentiation group was cultured in IMDM supplemented with 10 ml/L FBS, 20 ng/mL HGF, 10 ng/mL FGF-4,100 U/mL penicillin and 100 U/mL streptomycin. Each well was added 2 mL of the medium, and changed every 4 d. The medium was stored at -20 ℃ for albumin, alpha-fetoprotein (AFP) and urea assay.

Radioimmunoassay

Concentrations of AFP and albumin in the changed medium were determined by radioimmunoassay on d 4, 8, 12, 16, 20, 24, and 28. Since there might be a very small amount of AFP and albumin in the changed medium, the medium was condensed 5 times by freeze drying before radioimmunoassay.

Urea assay

Cell culture media at the indicated time points after the induction of hepatogenic differentiation were collected, analyzed for urea as previously described by Buga *et al*[25]. and optical densities were measured at 492 nm using a spectrophotometer. The control group was used as a negative control, which was cultured in IMDM supplemented with 10 mL/L FBS, 100 U/mL penicillin and 100 U/mL streptomycin.

Immunocytochemistry

A slide was put in each well. Cells on the slides were fixed with 4% paraformaldehyde at 20 ℃ for 10 min and permeabilized with 0.3% Triton X-100 in PBS for 10 min. Cells were incubated with 3 mL/L hydrogen peroxide in methanol for 10 min to block endogeneous peroxidase activity and then washed twice in PBS for 5 min. Nonspecific binding sites were blocked with 3 mL/L normal goat serum. Cells were incubated overnight at 4 ℃ with primary antibody (mouse anti-human monoclonal antibody of CK-18, 1:200). After washed twice for 15 min in PBS, slides were incubated at 37 ℃ for 30 min with secondary antibody (goat anti -mouse IgG marked by biotin), with streptoavidin marked by horseradish peroxidase, and then developed color with DAB, 0.01 mol/L PBS was substituted for primary antibody as the negative control. The positive cells presented as brownish yellow.

Periodic acid-Schiff treatment for glycogen

In the hepatocyte differentiation group, on d 16, 20, 24, and 28, we took out the medium from the flasks, rinsed cells 3 times with PBS , fixed them with 100 mL/L formaldehyde for 30 min. The cells were oxidized in 10 g/L periodic acid for 10 min and rinsed three times in dH_2O. Then, the cells were treated with Schiff's reagent for 10 min, rinsed in dH_2O for 10 min, stained with hematoxylin for 2 min, differentiated by 1% alcohol/ HCL, rinsed in dH_2O again and observed under an invert

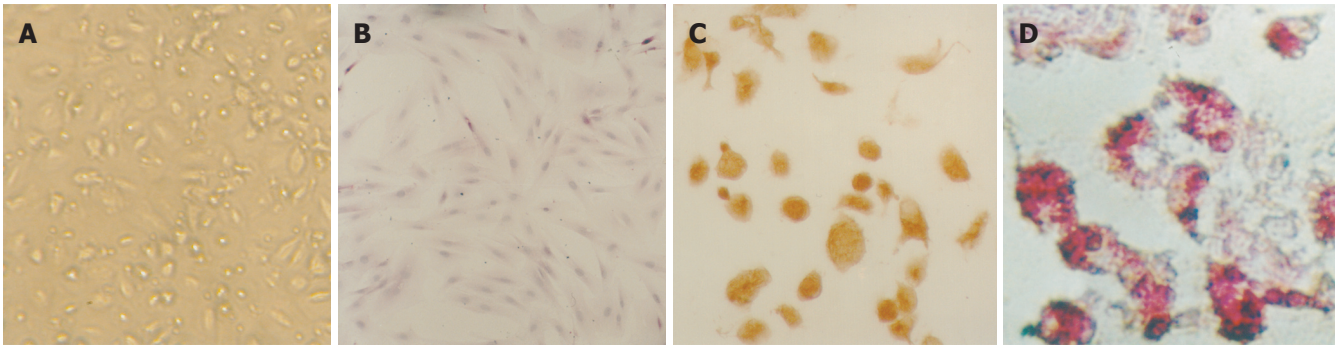

Figure 1 Different morphology and differentiation of HUCB-derived MSCs. **A**: morphology of primary cultured HUCB-derived MSCs. MSCs are spindle-like and osteroclasts are big and round; **B**: morphology of sub-cultured HUCB-derived MSCs stained with HE. The fibroblast-like cells are growing as a whirlpool (×100); **C**: representative immuocytochemistry showing expression of CK-18 on HUCB-derived MSCs on d 28 (SABC×200); **D**: cells stained by PAS. Glycogen storage is seen as accumulated magenta staining, the round and epithelioid cells show magenta staining in the region (×400).

Table1 Concentrations of AFP, albumin and urea in the medium at different time points (mean±SE)

Group	4 d	8 d	12 d	16 d	20 d	24 d	28 d
				AFP (μg/L)			
Differentiation	15.92±0.76	15.72±1.06	18.20±1.16[a]	20.57±1.98[a]	38.53±6.07[a]	39.47±4.06[b]	54.28±3.11[b]
Control	15.77±0.72	14.30±0.69	14.67±1.10	13.72±0.40	16.57±1.03	14.13±1.11	15.67±0.93
				Albumin (μg/mL)			
Differentiation	–	–	0.63±0.07	1.02±0.15[b]	1.45±0.21[b]	3.08±0.35[b]	3.63±0.30[b]
Control	–	–	0.40±0.12	0.20±0.06	0.15±0.06	0.22±0.07	0.25±0.10
				Urea (μmol/L)			
Differentiation	–	–	–	–	4.72±1.03[b]	7.27±0.89[b]	10.28±1.06[b]
Control	–	–	–	–	0.47±0.30	0.87±0.43	0.55±0.26

[a]P<0.05 *vs* control, [b]P<0.01 *vs* control, – stands for what could not detected. n = 6

microscope.

Statistical analysis

The data of AFP, albumin and urea were expressed as mean±SE. The statistical software SPSS12.0 was used. The results were analyzed by t-test. P<0.05 was considered statistically significant.

RESULTS

Isolation and culture of MSCs from HUCB

The cells isolated by gradient density centrifugation showed heterogeneity during the first five days. Adherent cells were observed on d 9-12. Spindle-shape cells appeared at the bottom of culture flasks. In addition, some big and round osteroclasts were observed among the spindle-like cells. Many round cells were suspended in the medium (Figure 1A). By changing the medium, the suspending cells became less and less. After the medium was changed twice, the suspending cells were completely removed from the medium, but osteroclasts still existed in the cells. The adhered cells were fibroblast-like and grew as a whirlpool. The sub-cultured cells were much pure and fibroblast-like, the osteroclasts disappeared (Figure 1B). The primary culture cells reached confluence 10-12 d later, the cells sub-cultured at a ratio of 1:3 reached confluence 8-10 d later. Using the method, we isolated HUCB-derived MSCs from 10 samples of 39, which were isolated within 6 h. The rate of separation was 25.6%. MSCs could not be isolated from the other sample.

MSCs became small, round and epithelioid cells after cultured with FGF-4 and HGF

When the cultured cells reached 70% confluence, they were treated with hepatocyte differentiation medium, containing IMDM supplemented with 10 mL/L FBS, 20 ng/mL HGF, 10 ng/mL FGF-4, 100 U/mL penicillin and 100 U/mL streptomycin. Before the 8-d treatment they did not show any change compared to the controls. After treatment, small round cells appeared in the treatment group, epithelioid cells were also observed. The diameter of the cells was 12-16 μm. On d 28, approximately 63.6% of the cells were small, round and epithelioid. The control cells were still fibroblast-like, the density of the cells increased. The cells were overlapped in some regions.

Radioimmunoassay

By radioimmunoassay, albumin and AFP production was measured at various time points throughout the differentiating process. We could detect low levels of AFP on undifferentiated HUCB-derived MSCs, but not of albumin. However, there was a small amount of albumin in the medium, which was hardly detected by

albumin RIA. Compared to controls, the levels of AFP increased significantly from d 12 with a concentration of 18.20±1.16 μg/L ($P<0.05$) in MSCs cultured with FGF-4, and HGF continued to increase and was higher on d 28 ($P<0.01$) with a concentration of 54.28±3.11 μg/L. The level of albumin could not be detected by radioimmunoassay before d 12, albumin increased significantly on d 16 with a concentration of 1.02±0.15 μg/mL ($P<0.01$), and to 3.63±0.30 μg/mL on d 28 ($P<0.01$). According to the data, increasing time points were different between AFP and albumin (Table 1).

Urea assay

Urea production and secretion by hepatocytes were detected at various time points throughout the differentiating process. Following treatment with FGF-4 and HGF, urea produced by MSCs was detected with a concentration of 4.72±1.03 μmol/L on d 20, and increased to 10.28±1.06 μmol/L on d 28 (Table 1).

Immunocytochemistry

On d 8, 16, 24, and 28, we took out the cells on the slides respectively. The cells expressed CK-18 on d 16 (Figure 1C). The control group was negative.

Periodic acid-Schiff treatment for glycogen

We analyzed the levels of glycogen storage by periodic acid-Schiff (PAS) staining of FGF-4 and HGF-induced MSCs on d 20, 24, and 28. Glycogen storage was seen on d 24, being positively stained for PAS (Figure 1D).

DISCUSSION

There are many hematopoietic stem cells and MSCs in HUCB. Many methods can isolate MSCs from HUCB[26-28]. In this study, we used the Ficoll (1.077 g/mL) to isolate MSCs from HUCB, we combined the gradient density centrifugation with plastic adherence and changed the medium many times to purify MSCs after gradient density centrifugation. This method is relatively simply, and can easily get pure MSCs. We isolated MSCs from 10 of 39 samples, which is in accordance with the previous study[28]. MSCs should be isolated from the sample as soon as possible. Our results showed that it was difficult to isolate MSCs from the samples, because the activity of MSCs is low, even loses with time prolonged.

During the 28 d of differentiation, FGF-4 and HGF induced MSCs into cells with morphological and functional characteristics of hepatocytes. In our study, the cells that differentiated into hepatocyte-like cells could produce urea, secrete albumin, AFP and Ck-18, and store glycogens. Urea production was characterized by hepatocyte activity, although kidney tubular epithelium also produced urea. In contrast, albumin, AFP and Ck-18 production is a specific test for the presence and metabolic activity of hepatocytes. Only hepatocytes can generate and store glycogens. In our research, we found that AFP could be detected throughout the differentiating process because the medium contained a low level of AFP. From d 12, the level of AFP increased significantly compared to controls, suggesting that MSCs can secrete AFP. It was reported that AFP is produced by immature hepatocytes. That is to say, hepatocytes are immature on d 12. Before d 12, the concentration of albumin could not be measured by radioimmunoassay because the amount of albumin was small in the medium. When MSCs differentiated, albumin was significantly secreted by MSCs from d 16, CK-18 was expressed by cells from d 16, and urea was significantly secreted from d 20. By periodic acid-Schiff (PAS) staining, the differentiated cells could store glycogens. The data suggest that HUCB-derived MSCs can differentiate into hepatocytes by induction of FGF-4 and HGF.

HGF was first identified as a blood-derived mitogen for hepatocytes. HGF and its receptor c-Met are the key factors for liver growth and function. After cultured with only HGF, adult human bone marrow MSCs could also differentiate into hepatocytes *in vitro*[29]. FGF-4 is mitogenic for fibroblasts and endothelial cells. Mouse embryonic stem cells grown in medium supplemented with FGF-4 could differentiate into cells expressing hepatocyte-specific genes and antigens[30]. By cooperation of HGF and FGF, the differentiation of MSCs could be triggered and MSCs could develop into hepatocytes.

Our experimental data showed the hepatic differentiation potential of HUCB-derived MSCs. HUCB-derived MSCs have more advantages over bone marrow MSCs. It is suggested that HUCB-derived MSCs are a source of cell types for cell transplantation therapy of liver diseases.

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