Biochemical and Biophysical Research Communications 422 (2012) 539–545

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

In vivo hepatic differentiation of mesenchymal stem cells from human umbilical cord blood after transplantation into mice with liver injury

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ARTICLE INFO

Article history:
Received 20 April 2012
Available online 9 May 2012

Keywords:
Hepatic differentiation
Mesenchymal stem cells
Umbilical cord blood
Transplantation
Liver injury

ABSTRACT

Aim: The aim of this study was to analyze the hepatic differentiation potential of human umbilical cord blood-derived mesenchymal stem cells (hUCBMSCs) after transplantation into severe combined immune deficiency (SCID) mice with liver injury induced by D-galactosamine/lipopolysaccharide (GalN/LPS) and to explore the possibility that cells can partially repair GalN/LPS-induced hepatic damage.
Methods: Mononuclear cells (MNCs) were isolated from fresh human umbilical cord blood, characterized by flow cytometry, and then transplanted into GalN/LPS-injured mice. Specimens were collected at 7, 14, 21, and 28 days after hUCBMSC transplantation. Histopathological changes were analyzed by hematoxylin and eosin staining. Polymerase chain reaction (PCR) for a specific marker of human cells, the human Alu sequence, was performed to locate exogenous hUCBMSCs in mouse livers. Expression of human hepatocyte-specific markers such as human albumin (hALB), human alpha-fetoprotein (hAFP), human cytokeratin 18 (hCK18), and human cytokeratin 19 (hCK19) were analyzed by reverse transcriptase (RT)-PCR and immunohistochemical staining.
Results: The hUCBMSCs were positive for the human MSC-specific markers CD271, CD29, CD90, CD105, and CD73, but negative for CD31, CD79b, CD133, CD34, and CD45. Histological findings showed that the hepatic damage in mice was attenuated after hMSC administration, and liver architecture was much better preserved. Human cells in the injured liver of recipient mice were detected by PCR for the human Alu sequence. In addition, expression of markers of hepatic lineage, including hALB, hAFP, hCK18, and hCK19, was detected by immunohistochemistry and RT-PCR in mouse livers after hUCBMSC transplantation, suggesting the formation of hepatocyte-like cells in vivo.
Conclusion: MSCs from hUCB exhibit the potential to differentiate into hepatocyte-like cells in the livers of hUCB-transplanted mice as well as partially repair the liver damage induced by GalN/LPS.

1. Introduction

Mesenchymal stem cells (MSCs) represent an archetype of multipotent adult stem cells that are being explored as a promising new treatment in stem cell-based liver regenerative medicine [1–3]. Currently, most applications and clinical trials involve MSCs from bone marrow (BM-MSCs). However, the technique of bone marrow collection remains invasive, and the frequency and differentiating potential of BM-MSCs decrease significantly with age [4]. Other tissues besides bone marrow, including human umbilical cord blood (UCB) [5,6], have recently been shown to provide an alternative source for MSCs. UCB-derived MSCs (UCB-MSCs) are similar to BM-MSCs with respect to cell characteristics and multilineage differentiation potential [7–9]. In addition, UCB-MSCs have clinical advantages because of their accessibility, low risk of viral contamination, painless procedures for donors, and less pronounced immune response [10]. Thus, UCB should be studied as a fascinating source of MSCs in stem cell-based therapy against liver disease. In various animal disease models, UCB-MSCs were found to attenuate lung injury in mice [11], protect the brain after trauma in mice [12], promote cutaneous wound healing [13], and show effectiveness in bone repair [14]. UCB-MSCs have also been used in animal models of myocardial infarction [15], cerebral ischemia [16], gliomas [17], acute spinal cord injury [18], and Alzheimer's disease [19]. A recent report showed that MSCs had the capacity to improve the survival rate in a rat model of acute

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http://dx.doi.org/10.1016/j.bbrc.2012.04.156

hepatic necrosis [20]. In vivo experiments have also demonstrated that transplanted UCB-MSCs were able to improve glucose homeostasis in rats with liver cirrhosis [21].

In the current study, we isolated MSCs from human UCB and characterized these cells in terms of their phenotypic profile. MSCs were then transplanted via the tail vein into severe combined immune deficiency (SCID) mice with D-galactosamine/lipopolysaccharide (GalN/LPS)-induced fulminant hepatic failure (FHF). After transplantation, the homing of MSCs to the injured liver and their differentiation into hepatocytes were investigated.

2. Materials and methods

2.1. MSC isolation and culture

After informed consent was obtained, human UCB samples were collected from donors at the Hangzhou Red Cross Hospital in China, using syringes containing heparin as an anticoagulant. The study protocol was approved by the Research Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University. The UCB samples were diluted 2:1 with phosphate-buffered saline (PBS), and mononuclear cells (MNCs) were obtained using Ficoll-Paque (Ficoll-Paque PLUS; GE Healthcare, USA) density gradient centrifugation (1.077 g/cm^3). The MNCs were collected and seeded at a density of 1×10^6 cells/cm^2 in tissue culture flasks. The cells were allowed to adhere for 5–7 days, and non-adherent cells were washed out with medium changes. The adherent cells were cultured in special medium (MesenCult Human Basal Medium plus MesenCult Human Supplement; Stemcell Technologies, Inc., Vancouver, Canada) at 37 °C in a humidified atmosphere containing 5% carbon dioxide, with a change of medium every 3 days. When adherent cells reached approximately 70–80% confluence, they were detached with 0.25% trypsin/EDTA (Invitrogen, Carlsbad, CA, USA), washed with PBS, collected by centrifugation at 1000 rpm for 5 min, plated at a 1:2 dilution, and cultured under the same conditions.

2.2. Flow cytometry

For cell surface antigen phenotyping, third- to fifth-passage cells were harvested, suspended at a concentration of 1×10^6 cells/100 μl in PBS, and incubated with mouse anti-human monoclonal antibodies against CD271, CD29, CD90, CD105, CD73, CD31, CD79b, CD133, CD34, CD45, CD13, and CD166 (eBioscience, Inc., San Diego, CA, USA), all of which were conjugated with fluorescein isothiocyanate, phycoerythrin, or allophycocyanin. Labeled cells were assayed by two-color flow cytometry using a Cytomics FC 500 MPL (Beckman Coulter, Inc., Los Angeles, CA, USA) and analyzed with MXP software.

2.3. Mouse model of FHF and hUCBMSC treatment

All of the experimental protocols were approved by the Animal Ethics Committee of Zhejiang University. Male SCID mice (4–8 weeks old, 18–23 g) were purchased from the Laboratory Animal Center, Zhejiang University, China. The animals were housed individually in cages in an air-conditioned room at 25 ± 1 °C with a 12-h dark/light cycle. All animals received human care during the study and had free access to water and laboratory chow. Animal protocols were approved by the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University before the commencement of the study.

The mouse model of FHF was induced by intraperitoneal injection of a mixture of D-galactosamine (D-gal) and lipopolysaccharide (LPS) (0.5 mg/g and 1 ng/g, respectively, in 1 mL of saline). The animals were then randomly divided into two groups. Group I comprised hUCBMSCs/GalN/LPS mice (n = 20). These FHF model mice received hUCBMSCs by injection of 5×10^5 cells in 0.5 mL DMEM into the tail vein. Group II comprised DMEM/GalN/LPS mice (n = 20). These FHF model mice did not undergo hUCBMSC treatment, but received the same volume of DMEM. Mice were sacrificed at 7, 14, 21, and 28 days after hUCBMSC or DMEM injection. The livers were collected and either fixed in 4% formalin and embedded in paraffin, or immediately frozen in liquid nitrogen and stored at −80 °C.

2.4. Pathology and immunohistochemistry

Livers were removed from mice, fixed with formalin, embedded in paraffin, cut into 5-μm sections, and mounted onto slides for pathological and immunohistochemical analyses. For the former, sections were treated with hematoxylin and eosin (HE) staining for routine histology. For immunohistochemistry, the liver slides were heated in citrate buffer (0.02 mol/L, pH 5.8) for antigen retrieval. Endogenous peroxidase activity was prevented by incubation in 0.3% hydrogen peroxide in methanol for 15 min. Nonspecific binding was blocked by 5% bovine serum albumin in PBS. The sections were then incubated with diluted primary antibody against human albumin (ALB) (1:200), human alpha-fetoprotein (AFP) (1:250), human cytokeratin 18 (CK18) (1:500), or human cytokeratin 19 (CK19) (1:400) according to the manufacturer's instructions. The slides were washed and incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Peroxidase activity was visualized by exposure to diaminobenzidine tetrahydrochloride solution (DAB kit; Vector Labs) for 3–5 min. The sections were then washed, counterstained with hematoxylin for 1 min, dehydrated, mounted with coverslips, and analyzed by light microscopy (TE2000; Nikon, Japan). Normal human liver tissue obtained under informed consent provided positive controls.

2.5. Reverse transcriptase-polymerase chain reaction

To confirm the immunohistochemical findings, RT-PCR was performed. Total RNA of liver samples was isolated using Trizol reagent (Invitrogen). The quantity and purity of the RNA were estimated after measuring the absorbance at 260 and 280 nm. cDNA was synthesized from 1 μg of RNA using oligo(dT) primers with an Improm-II™ Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Human-specific primers were designed to detect the expression of human albumin, AFP, CK18, and CK19 mRNA in mouse liver tissues. GAPDH was used as the internal standard. The primer sequences are listed in Table 1. PCR was performed in a thermal cycler (Thermo Hybaid; Thermo

Table 1
Primers and conditions used for RT-PCR.

Primer	Sequence	Amplicon size (bp)	Cycle conditions
ALB	GAGCTGCTCCATCTGTAGGG-3′ 5′-CACAGTCTGCTGAGGTTGGA-3′	166	94 °C/30 s; 58 °C/30 s; 72 °C/60 s
CK18	5′-CTGAGCAAAGGCAATCAACA-3′ 5′-TGGCACAATGAAGTGGGTAA-3’	164	94 °C/30 s; 58 °C/30 s; 72 °C/60 s
CK19	5′-AGGTGGATTCCGCTCCGGGCA-3′ 5′-ATCTTCCTGTCCCTCGAGCA-3′	460	94 °C/60 s; 64 °C/60 s; 72 °C/120 s
AFP	5′-ACCATGAAGTGGGTGGAATC-3′ 5′-ATTTAAACTCCCAAAGCAGCAC-3′	1800	94 °C/120 s; 62 °C/120 s; 72 °C/180 s
GAPDH	5′-GGGTCGGAAGGATTCCTA-3′ 5′-GGTCTCAAACATGATCTGGG-3′	250	94 °C/60 s; 55 °C/30 s; 72 °C/60 s

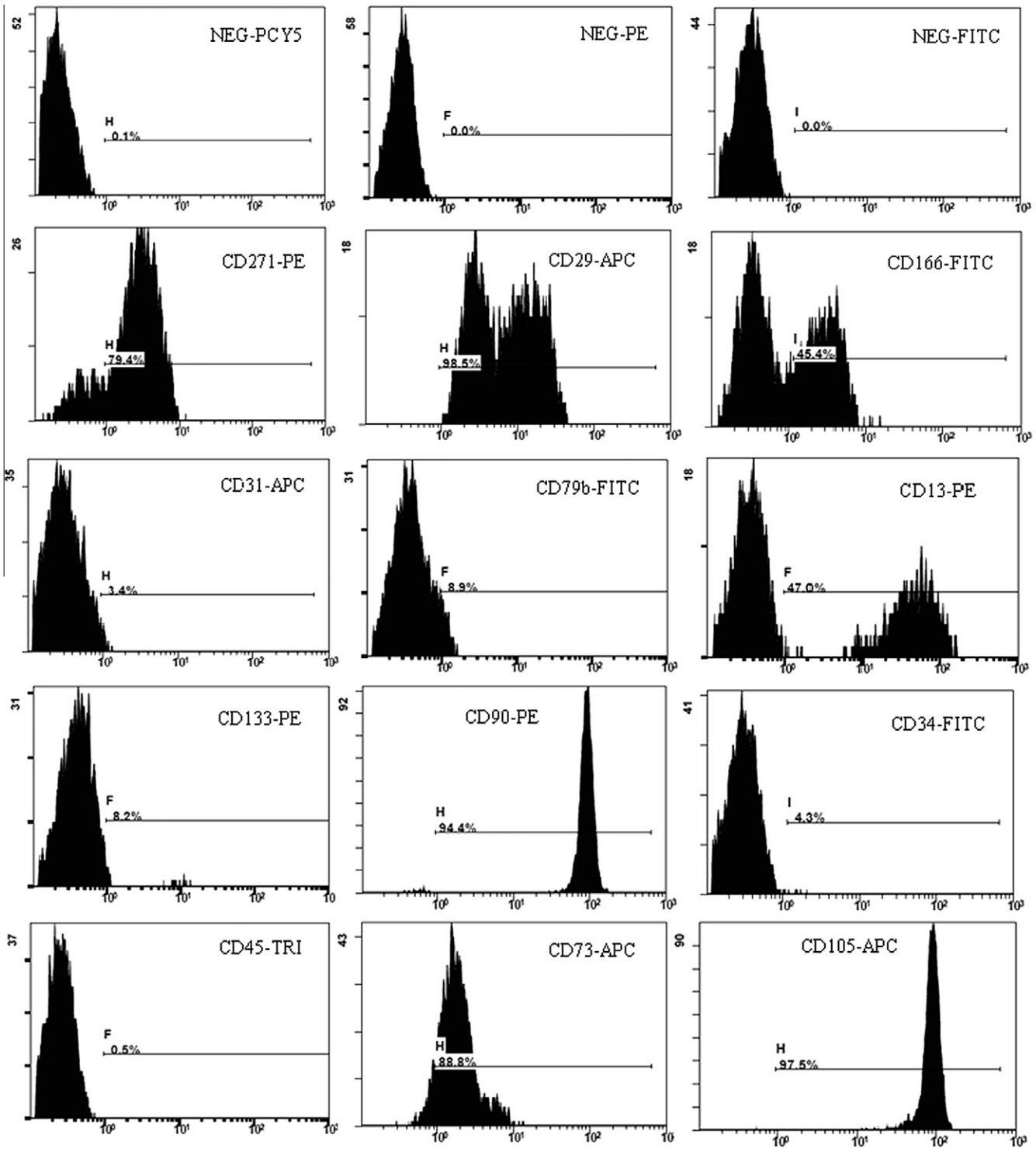

Fig. 1. Immunophenotype of hUCBMSCs. Cells were harvested at passages 3–5; labeled with antibodies against the human antigens CD271, CD29, CD166, CD31, CD79b, CD13, CD133, CD90, CD34, CD45, CD73, and CD105; and analyzed by flow cytometry.

Scientific, Waltham, MA, USA). Amplified PCR products were electrophoresed in a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and visualized under UV light.

2.6. Detection of human Alu sequence by PCR

To detect human hUCBMSCs in recipient mouse liver, PCR for the human Alu sequence was performed using total DNA extracted from the livers with a DNeasy Blood & Tissue Kit (Qiagen, USA) according to the manufacturer's instructions. The primers for the target were 5-CTGGGCGACAGAACGAGATTCTAT-3 and 5-CTCAC-TACTTGGTGACAGGTTCA-3, and the PCR reaction was performed as follows: denaturing at 94 °C for 2 min, amplification for 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and extension for 59 s at 72 °C. The PCR products were analyzed by fractionation in a 1.2% agarose gel and visualized with ethidium

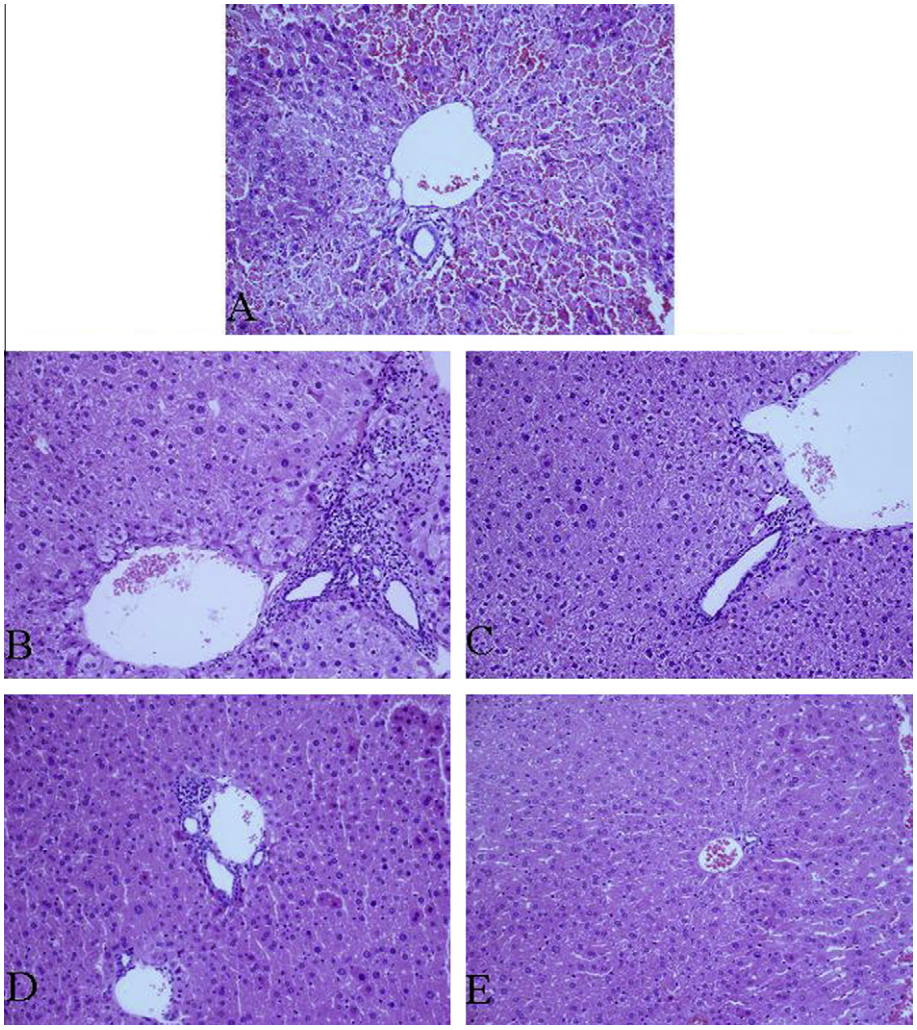

Fig. 2. Representative hematoxylin-eosin staining. (A) Group II (Day 2). (B–E) Group I (Days 7, 14, 21, and 28) (10×).

bromide staining. Images were captured using a gel documentation system.

3. Results

3.1. Immunophenotypic characterization of hUCB-derived MSCs

To characterize the adherent cell population derived from hUCB, a selection of surface markers was examined by flow cytometry. The UCB-derived cells were positive for mesenchymal progenitor/stem cell-related antigens such as CD271, CD29, CD90, CD105, and CD73, and were negative for CD31, CD79b, CD133, CD34, and CD45 (Fig. 1). The lack of expression of CD34 (a hematopoietic lineage marker) and CD45 (a leukocyte common antigen) indicated that these cell cultures were not of hematopoietic origin. In addition, only 45.4 ± 10.2% of the UCB-derived cells expressed CD166, while 26.2 ± 20.8% stained positive for CD13. Thus, the adherent cells derived from hUCB exhibited the phenotype of MSCs.

3.2. Pathology

Histological examination using HE staining revealed seriously damaged liver lobular architecture and cell structure in the livers after GalN/LPS administration in the control group (Fig. 2A). Livers exposed to GalN/LPS showed multiple and extensive areas of cellular necrosis with serious intralobular hemorrhage. Inflammatory cell infiltration, hepatocyte degeneration, and disordered hepatocyte cords were also observed. Compared with the damage in the control livers, less hepatic damage was seen in the livers of mice administered hMSCs, and liver architecture was much better preserved. On day 7, inflammatory cell infiltration was still observed. Fibroplasia was visible in the portal areas. In addition, the number of binucleated cells was increased, with deeper cytoplasmic staining, suggesting that cell regeneration had occurred in the injured liver (Fig. 2B). There were fewer infiltrated inflammatory cells and necrotic hepatocytes on days 14 and 21, and slight fibroplasia and cell regeneration were still observed (Fig. 2C,D). On day 28, degeneration, necrosis, and inflammatory cell infiltration had almost disappeared, as revealed by nearly normal liver lobular architecture (Fig. 2E).

3.3. Hepatocyte differentiation of infused hUCBMSCs in the mouse liver

To investigate whether hUCBMSCs are capable of undergoing hepatic differentiation in vivo, the liver specimens of the FHF mice were examined immunohistochemically for expression of the human liver-specific markers hAFP, hAlb, hCK18, and hck19. Representative immunohistochemical staining patterns for these markers are shown in Fig. 3. On day 7 in hUCBMSC-transplanted

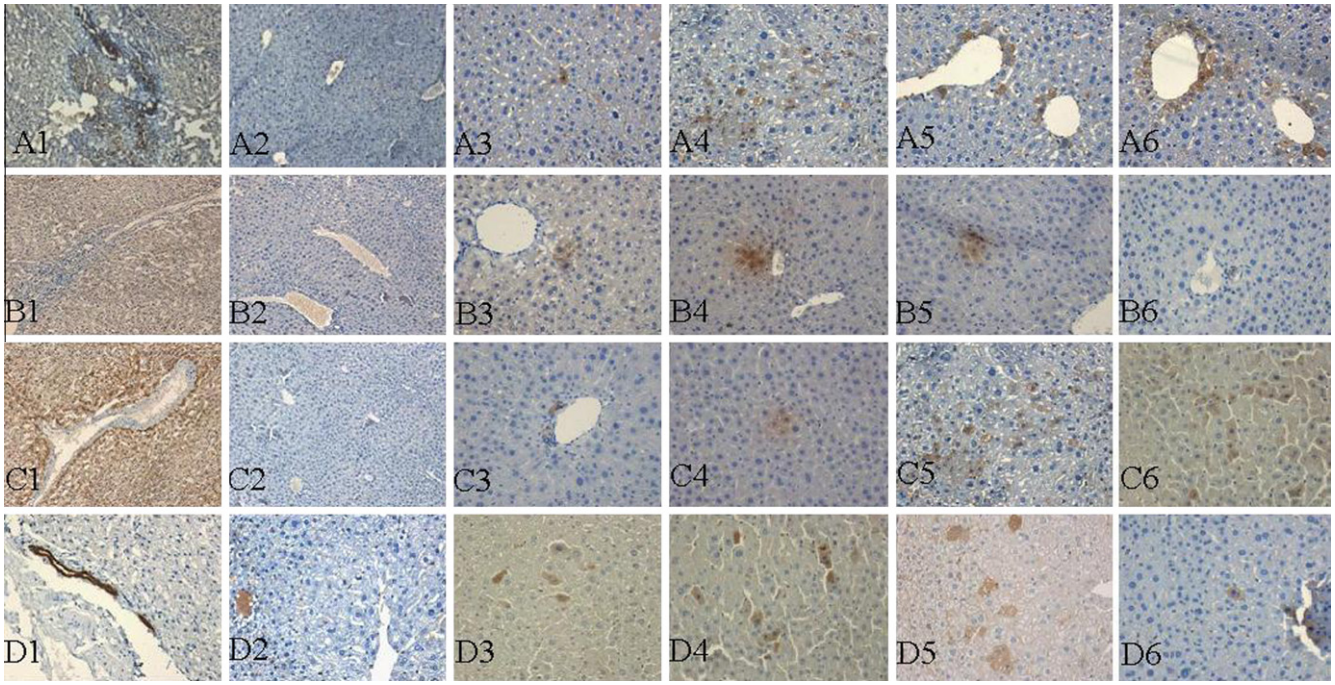

Fig. 3. Immunohistochemical staining. (A1–D1) Positive control (human liver tissue). (A2–D2) Immunohistochemical analysis of liver specimens from the control group. Sections from the untransplanted mice did not stain with antibodies specific for human ALB, AFP, CK18, or CK19. (A3–A6) Immunohistochemical staining for human ALB in Group I at 7, 14, 21, and 28 days after hUCBMSC transplantation. (B3–B6) Immunohistochemical staining for human AFP in Group I at 7, 14, 21, and 28 days after hUCBMSC transplantation. (C3–C6) Immunohistochemical staining for human CK18 in Group I at 7, 14, 21, and 28 days after hUCBMSC transplantation. (D3–D6) Immunohistochemical staining for human CK19 in Group I at 7, 14, 21, and 28 days after hUCBMSC transplantation.

mice, staining for hAFP and hCK19 became weakly positive in spots (Fig. 3B3,D3); staining for hAlb and hCK18 also became positive, but was more faint (Fig. 3A3,C3). All four markers were stained in the cytoplasm. On day 14, both $hAFP^+$ and $hCK19^+$ cells appeared to form a cluster with stronger staining intensity (Fig. 3B4,D4), and the number of cells staining positive for hAlb and hCK18 was increased, with more evident staining intensity (Fig. 3A4,C4). On day 21, $hAFP^+$ and $hCK19^+$ cells remained as small clusters, while $hAlb^+$ and $hCK18^+$ cells formed clusters with strong staining intensity (Fig. 3A5,B5,C5,D5). On day 28, staining for hAFP was negative (Fig. 3B6), and CK19 was barely detectable (Fig. 3D6), showing a single spotty positive stain. In contrast, the staining intensity for $hAlb^+$ and $hCK18^+$ cells was increased (Fig. 3A6,C6). No expression of any of the four markers was observed in the liver specimens of untransplanted mice (Fig. 3A1,B1,C1,D1).

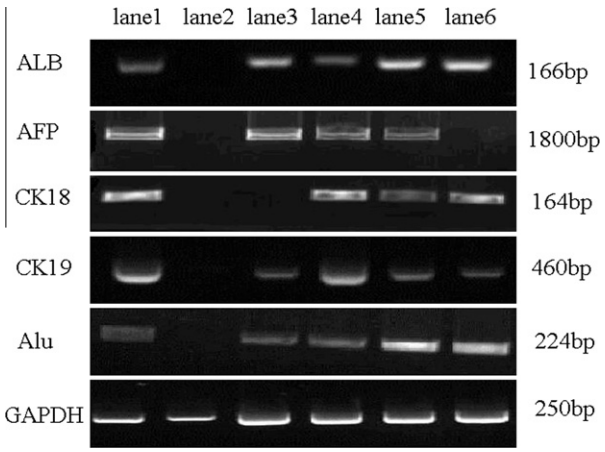

Fig. 4. mRNA expression of hAlb, hAFP, hCK18, and hCK19 as detected by RT-PCR, and human Alu sequence as detected by PCR. Lane 1, positive control (human DNA); Lane 2, control group; Lanes 3–6, Group I (7, 14, 21, and 28 days after hUCBMSC transplantation).

3.4. RT-PCR

To confirm that hUCBMSCs had undergone hepatocyte differentiation in the recipient livers, mRNA expression of hAlb, hAFP, hCK18, and hCK19 was determined by RT-PCR using specific primers for each (Fig. 4). On days 7–28 after injection, the expression of hAlb, hCK18, and hCK19 mRNA was detected in the liver of mice that had received hUCBMSCs. The expression of hAFP mRNA was detectable from 7 to 21 days after transplantation of hUCBMSCs, but had disappeared at day 28. None of the markers were detectable in the control group livers. The mRNA expression patterns of these markers were basically in accordance with the immunohistochemical results, verifying hepatic differentiation of transplanted hUCBMSCs in the recipient mouse liver.

3.5. Detection of transplanted human cells in the mouse liver

To investigate the engraftment capacity of hUCBMSCs into injured mouse liver, human-derived cells in recipient livers were detected by PCR using a human Alu-specific primer set. The human Alu sequence was detected in recipient livers at 7 days post-transplantation, and the level increased from day 14 to 28 (Fig. 4). This shows the presence and expansion of grafted human cells in the transplanted mice.

4. Discussion

In the present study, we isolated hUCBMSCs by gradient centrifugation, and a flow cytometric phenotype analysis revealed that the hUCBMSCs expressed MSC-related antigens, corroborating results from previous studies [22,23], and did not express hematopoietic cell antigens [24]. The undifferentiated hUCBMSCs were

injected into the tail vein of GalN/LPS-induced FHF SCID mice. The major finding of our study was that transplanted hUCBMSCs are able to migrate into injured liver, where they can differentiate into hepatocyte-like cells. In addition, hUCBMSCs improved the liver structure in experimental liver injury in mice. Thus, hUCBMSCs may be suitable as seed cells in cellular therapy for acute liver injury.

Gram-negative bacterial LPS is considered to have relevance in the development of FHF and multiple organ failure, by releasing a wide variety of inflammatory mediators. The administration of GalN together with LPS is frequently used to create animal models of FHF for the study of liver injury [25–27]. The simulation of FHF by GalN/LPS injection has been shown to result in a high mortality rate and severe hepatic damage, as reflected by significantly increased serum liver enzymes combined with serious hepatic injury [28,29]. We used this chemical liver injury model to investigate the effect of hUCBMSC transplantation on acute liver injury and to determine the in vivo hepatogenic potential of hUCBMSCs.

Pathological examination by HE staining of liver specimens from the GalN/LPS-injected FHF mice revealed obvious injury characteristics such as massive necrosis, serious intralobular hemorrhage, inflammatory cell infiltration, and hepatocyte denaturation. At 7 days after FHF mice were injected with hUCBMSCs, less hepatic damage was observed, and cell regeneration was evident. At day 28, HE staining showed nearly normal liver lobular architecture, and necrosis, hepatocyte denaturation, and inflammatory cell infiltration had almost disappeared. This demonstrates that transplantation of hUCBMSCs can partially ameliorate mouse hepatic injury induced by GalN/LPS.

The expression of hepatocyte-specific markers in liver samples from hUCBMSC recipient mice was investigated by RT-PCR and immunohistochemical staining, to determine whether the transplanted cells had differentiated into hepatocyte-like cells. The expression of hAlb, hCK18, and hCK19 was detected in the livers of FHF mice at 7–28 days after hUCBMSC transplantation; hAFP expression was no longer detected after 21 days. Immunopositive clusters or scattered isolated cells were observed in recipient mouse livers. These results suggest that hUCBMSCs can differentiate into hepatocyte-like cells in GalN/LPS-injured mouse livers.

Recent research has indicated that MSCs possess the ability to home to injured organs after transplantation [30–32]. In rats with CCl_4-induced liver injury, the number of transplanted MSCs that homed to the liver was related to the presence of liver injury, and was not related to the site of cell injection, whether through the tail vein or other routes such as the portal vein [30,33]. MSCs are reportedly able to overcome immune rejection to home to injured regions [8,34]. In the current study, the homing of hUCBMSCs to the injured liver was confirmed by the presence of the human Alu sequence, as detected by PCR, in the injured liver tissue of recipient mice at 7–28 days after transplantation.

In the present study, we demonstrated that infused hUCBMSCs can exert a protective effect against hepatic injury and have the potential for in vivo hepatic differentiation, suggesting that these cells may provide a new approach for cell therapy in hepatic diseases. The exact mechanisms by which MSCs repair liver injury and by which hepatic differentiation occurs remain to be elucidated in future studies.

Acknowledgments

We thank the staff and patients of Hangzhou Red Cross Hospital for providing the umbilical cord blood samples. We are grateful to Dr. Da Yu and Mr. Hui Cheng for their excellent technical help with the animal models.

This work was financially supported by the Chinese High Tech Research & Development (863) Program (No. 2011AA020104), Science Fund for Creative Research Groups of the National Natural Science Foundation of China (No. 81121002), and the Fundamental Research Funds for the Central Universities.

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