Human Umbilical Cord Matrix Stem Cells Efficiently Rescue Acute Liver Failure Through Paracrine Effects Rather than Hepatic Differentiation

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There is increasing evidence that mesenchymal stem cells (MSCs) derived from different tissues could act as an alternative source of mature hepatocytes for treatment of acute liver failure (ALF). Human umbilical cord matrix stem cells (hUCMSCs) represent a novel source of MSCs. We examined the therapeutic potential and the different mechanisms of hUCMSCs by their transplantation into nonobese diabetic severe combined-immunodeficient (NOD-SCID) mice with carbon tetrachloride (CCl₄)-induced ALF in comparison to adult human hepatocytes (AHHs). The characteristics of isolated hUCMSCs were determined from MSCs and hepatocyte marker expression, hepatic function, and differentiation. Native hUCMSCs constitutively expressed some hepatic markers, though weaker hepatocyte-specific functions were observed when compared to AHHs. When native hUCMSCs or AHHs were transplanted into livers of NOD-SCID mice with ALF induced by CCl₄, both hUCMSCs and AHHs provided a significant survival benefit and prevented the release of liver injury biomarkers. hUCMSCs were found to engraft within the recipient liver and differentiated into functional hepatocytes, whereas the HepPar1-/albumin (ALB)-positive cells of the hUCMSC group were less than the AHH group in the recipient liver. Higher values of human ALB in the serum of mice-transplanted AHHs were determined in comparison with levels in mice-transplanted hUCMSCs. The analysis of mouse serum cytokine levels showed that hUCMSC transplantation was even more effective than treatment with AHHs and successfully down-regulated the systemic inflammatory cytokines such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , IL-6, IL-10, and IL-1 receptor antagonist (IL-1RA). Furthermore, paracrine effects produced by hUCMSCs were identified by indirect coculture with damaged mouse hepatocytes (MHs) induced by CCl₄. Coculture with hUCMSCs significantly increased the viability, ALB secretion of damaged MHs, and greatly enhanced the regeneration of MHs *in vitro* when compared with AHHs. These data suggest that direct transplantation of native hUCMSCs can rescue ALF and repopulate livers of mice through paracrine effects to stimulate endogenous liver regeneration rather than hepatic differentiation for compensated liver function, which is the primary effect of AHHs. Thus, hUCMSCs can be a potential alternative source of AHHs for cell therapy of ALF and eliminate the shortage of hepatocytes.

Introduction

HEPATOCYTE TRANSPLANTATION HAS been proposed as a tangible alternative to liver transplantation for treatment of acute liver failure (ALF) because of the simpler and less invasive procedure.¹ However, the procurement of transplantable hepatocytes is hampered by the paucity of cadaveric livers, the limited replicative potential, and the

concomitant loss of hepatic functions upon *in vitro* culture.² In this scenario, stem cells are particularly attractive because of their capacity for self-renewal and multipotency. Embryonic stem cells and induced pluripotent stem cells offer immense potential for regenerative medicine, but their use remains debatable because of their low differentiation efficiency as well as the risk of tumorigenesis.^{3–5} Mesenchymal stem cells (MSCs) are excellent candidates for cell therapy.

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Several investigators have demonstrated the capacity of MSCs to differentiate into hepatocyte-like cells, and the majority of these were isolated from bone marrow, adipose tissue, and umbilical cord blood.^{6–8} However, the numbers of MSCs from bone marrow and adipose tissue decrease significantly with age.^{9–11} MSCs with hepatogenic capacities have also been isolated from umbilical cord blood,⁸ but the yield of isolated cells is highly dependent in blood quality and remains limited compared with bone marrow.¹² Recently, several studies have focused on human umbilical cord matrix stem cells (hUCMSCs) as a novel source with clear advantages, such as a noncontroversial and inexhaustive source, lower immunogenic potency, higher proliferative activity, and multipotency.^{13–16}

Several studies have reported that MSCs derived from different tissues could be an alternative source of hepatocytes for treatment of ALF.^{17–19} hUCMSCs represent a unique type of MSCs isolated from umbilical cord that is normally discarded after a live birth.^{20,21} To the best of our knowledge, however, there have been no study to investigate whether and how undifferentiated hUCMSCs are able to repopulate livers of mice with ALF. Moreover, the difference between hUCMSCs and adult human hepatocytes (AHHs) was not considered and evaluated in the therapeutic application of ALF. We speculate that undifferentiated hUCMSCs in damaged livers may exhibit multiple advantages of hepatic differentiation, hepatic function, paracrine effects, and possible immunoloregulation.^{22–24}

In this study, we evaluated hepatic markers and hepatocyte-specific functions of native hUCMSCs *in vitro* compared with AHHs. Furthermore, the therapeutic potential of native hUCMSCs and AHHs was comparatively analyzed through direct transplantation to rescue carbon tetrachloride (CCl₄)-induced ALF of nonobese diabetic severe combined-immunodeficient (NOD-SCID) mice *in vivo*. Finally, paracrine effects produced by hUCMSCs and AHHs were identified by indirect coculture with damaged mouse hepatocytes (MHs) induced by CCl₄. Our study provides new insights into the substitution of AHHs to reverse ALF by native hUCMSCs.

Materials and Methods

Isolation and expansion of hUCMSCs

This study was approved by the Research Ethics Committee of the Southwest Hospital and Third Military Medical University. With the written consent of the parents, umbilical cords were obtained from delivering full-term infants. hUCMSCs were isolated as described previously.²⁵ The cell pellet was resuspended in an expansion medium composed of the Dulbecco's modified Eagle's medium low glucose (DMEM-LG) (1 g/L D-glucose; Invitrogen) with sodium pyruvate and L-glutamine supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, and 100 µg/mL streptomycin. Plated cells were cultured in the expansion medium at 37°C and 5% carbon dioxide in a fully humidified atmosphere.

Immunophenotyping of MSC-like cells by flow cytometry

Native hUCMSCs (at passage 4) were trypsinized by TrypLE Express (Invitrogen) and suspended at a concentra-

tion of 1×10^6 cells/mL in phosphate-buffered saline (PBS) containing 0.1% FBS and then incubated at 4°C with antibodies against MSC markers (CD90, CD73, CD105, CD13, CD54, and CD166), hematopoietic cell markers (CD45, CD34, CD14, CD117, and CD19), integrins, and receptors for extracellular matrix (CD29 and CD44) and major histocompatibility (HLA-ABC and HLA-DR) (all purchased from eBioscience). After 30 min, cells were washed and resuspended in 300 µL Cell Fix (BD). Flow cytometry with FACSCalibur (BD) was performed.

Adipogenic and osteogenic differentiation

Adipogenic and osteogenic induction was performed as described previously.²⁵ After 4 weeks of induction, the cells were stained using the Oil Red O procedure or the Alizarin Red solution to detect the presence of neutral lipid vacuoles in adipocytes or calcium deposition in osteocytes, respectively. The adipogenic-specific genes, including lipoprotein lipase (LPL), peroxisome proliferator-activated receptor γ (PPAR- γ), and adiponectin, as well as the osteogenic-specific genes (osteopontin, osteonectin, and collagen I), were further detected by reverse transcriptase-polymerase chain reaction (RT-PCR) as described below. The appropriate primers are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertonline.com/tea).

Hepatogenic differentiation

For hepatogenic differentiation, hUCMSCs (from passage 3 to passage 6) were seeded at a density of 1.0×10^4 cells/cm² in 6- or 24-well plates coated with 5 µg/cm² of rat tail collagen type I (BD Biosciences) in DMEM-LG supplemented with 10% FBS and 100 U/mL penicillin and 100 µg/mL streptomycin. Differentiation was induced by treating hUCMSCs with a step-1 differentiation medium, consisting of the Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 20 ng/mL epidermal growth factor and 10 ng/mL basic fibroblast growth factor for 2 days. Thereafter, differentiation was induced by treating hUCMSCs with 10 ng/mL basic fibroblast growth factor, 20 ng/mL hepatocyte growth factor (HGF), 0.61 g/L nicotinamide (Sigma), and 1% insulin-transferrin-selenium (ITS) premix for 10 days. The maturation step consisted of treatment with the IMDM containing 20 ng/mL oncostatin M, 1 µmol/L dexamethasone, and 1% ITS premix for 10 days. For each step, the medium was changed every 3 days. All growth factors were bought from Invitrogen.

Isolation and culture of AHHs

Human hepatocytes were isolated from surgical liver resections using a two-step collagenase perfusion procedure as previously described.²⁶ The hepatocyte culture medium consisted of the DMEM supplemented with 10% FBS, 14 ng/mL glucagon, 0.5 U/mL insulin (Invitrogen), 7.5 µg/mL hydrocortisone, 200 µg/mL streptomycin, and 200 U/mL penicillin.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from cultured cells using an RNeasy Mini Kit (Qiagen) and was treated with RNase-Free DNase (Qiagen). RT-PCR was carried out with the Qiagen OneStep RT-PCR Kit, according to the manufacturer's

instructions. Specific primers and annealing temperature were used for PCR amplifications (Supplementary Table S1). All PCR experiments were performed at 30 cycles. Samples were electrophoresed on a 1% agarose gel, and nucleic acids were visualized by ethidium bromide staining.

Immunocytochemistry

Cells were fixed in 4% formaldehyde for 10 min, followed by incubation with Protein Block for 30 min. Undifferentiated hUCMSCs and AHHs were analyzed by immunohistochemistry with α -fetoprotein (α -FP, 1:300), albumin (ALB, 1:250; Bethyl Laboratories), cytokeratin-19 (CK-19, 1:200; Invitrogen), cytokeratin-18 (CK-18, 1:200; Invitrogen), HepPar1 (1:200, Invitrogen), and hepatocyte nuclear factor 4 α (HNF-4 α , 1:200; Invitrogen) antibodies overnight at 4°C. The rhodamine (1:1000) or fluorescein isothiocyanate (FITC) (1:1000)-conjugated secondary antibodies were applied for 30 min. Nuclei staining was performed using 4,6-diamidino-2-phenylindole (Sigma-Aldrich). Immunoperoxidase staining was performed as previously described.²⁵

Periodic acid-Schiff staining

Cells were fixed with 10% formaldehyde oxidized in 1% periodic acid (Sigma-Aldrich) for 10 min and rinsed twice with water. Afterward, cells were treated with the Schiff's reagent for 10 min and rinsed with water.

Urea assay

AHHs and undifferentiated hUCMSCs were cultured for 24 h in the DMEM supplemented with or without 0.5 mmol/L ammonium chloride. Supernatants were collected and preserved at -20°C until further investigation. Urea concentrations were determined by a colorimetric assay (Sigma-Aldrich) according to the manufacturer's instructions. The DMEM was used as a negative control.

Animal model and cell transplantation

NOD-SCID mice were purchased from the Laboratory Animal Center, Third Military Medical University. All procedures follow the ethical guidelines and were approved by the Institutional Animal Care and Use Committee of the Third Military Medical University. Animal model and cell transplantation were performed as described previously.¹⁷ CCl₄ (Sigma-Aldrich) was dissolved in vegetable oil at 10% concentration and administered to animals by gavage at a dose of 0.28 mL/kg. Cells were transplanted under ethyl ether inhalation anesthesia at 18 h after administration of CCl₄. Direct intrasplenic injections were made after laparotomy. Three transplantation groups were designed: group A, control mice that received 100 μ L of PBS injected into their spleen ($n=11$); group B, mice that received 100 μ L of hUCMSCs (4×10^7 cells/kg) injected into their spleen ($n=12$); and group C, mice that received AHHs (4×10^7 cells/kg) injected into their spleen ($n=11$). After the transplantation, blood samples were collected, and the entire livers were taken, fixed, and prepared every 14 days for further analysis from sacrificed mice.

Serum transaminase levels

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in mouse blood were measured with an automated biochemical analyzer.

Histologic and immunohistochemical analysis

Livers were fixed in 4% formaldehyde for 24 h and embedded in paraffin. Five-micrometer-thick liver sections were deparaffinized and fixed. For histology, sections were stained with hematoxylin and eosin (H&E) (Sigma-Aldrich). Endogenous peroxidase activity was prevented by a bath of methanol and 0.3% hydrogen peroxide for 15 min. Antigens were retrieved by incubating the section in a warm (97°C) 0.02-mol/L citrate buffer (pH 5.8). After washing, nonspecific immunostaining was prevented by 1-h incubation in PBS 1% bovine and serum albumin. Tissues were incubated overnight at 4°C with rabbit anti-human antibodies against ALB (1:3000), α -FP (1:1000), and HepPar1 (1:2000), or with the mouse anti-human CK-18 antibody (1:150). Detection was performed after incubating the sections with horseradish peroxidase-conjugated antibodies (1:500). Peroxidase activity was revealed by a 5-min exposure to diaminobenzidine (Sigma-Aldrich). After washing, preparations were counterstained with Mayer's hematoxylin for 10 min and mounted for analysis.

ALB assay

The ALB concentration of culture supernatants and serum of mice were screened by an enzyme-linked immunosorbent assay (ELISA). ELISA was performed as previously described.²⁷ Human and mouse ALBs secreted into the medium were determined by a human or mouse ALB ELISA quantitation kit (Bethyl Laboratories).

Cytokine levels

Serum samples were collected from NOD-SCID mice at 36 h after PBS, hUCMSC, or AHH transplantation. Quantification of serum levels of interleukin (IL)-1 β , IL-6, tumor necrosis factor- α (TNF- α), IL-10, HGF, and IL-1 receptor antagonist (IL-1RA) was determined by ELISA as per manufacturer's instructions (R&D Systems).

MH injury in vitro by CCl₄-induction and the recovery of cell viability and mouse ALB secretion

MHs were isolated from NOD-SCID mice by a two-step collagenase perfusion procedure.²⁸ MHs were seeded in the lower level of transwell chambers at a density of 1×10^5 cells/cm² in a hepatocyte medium as previously described. On day 2, CCl₄, diluted with absolute ethanol before addition to the medium, was supplemented into the DMEM at a final concentration of 8 mmol/L.²⁹ At 6 h, after addition of CCl₄, 1×10^5 hUCMSCs or AHH preseeded in transwell chambers were placed into the well containing CCl₄-treated hepatocytes for subsequent coculture. An empty transwell chamber served as the non-cocultured control. Viability and proliferation of MHs were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 24 h of coculture, and the results were expressed as relative viabilities to hepatocytes incubated without CCl₄. The supernatant samples were collected and preserved at -20°C for the mouse ALB assay.

MH proliferation in the damaged hepatocyte model in vitro

After induction of hepatocyte injury, cocultured cells were incubated with 10 μ M of the DNA synthesis marker,

bromodeoxyuridine (BrdU; Sigma-Aldrich). The MH proliferation was determined by counting the BrdU- and ALB-positive cells as previously reported.³⁰ At 48 h after coculture, MHs were fixed in 70% ethanol for 45 min and treated with 4 N HCl and 0.2% Triton X-100. After incubation in a blocking buffer for 30 min, cells were incubated for 60 min with anti-BrdU-Alex594 (Invitrogen) and goat anti-mouse ALB (Bethyl Laboratories) at 37°C, followed by FITC-conjugated anti-goat immunoglobulin G at room temperature. BrdU-positive cells among hepatocytes were counted in fluorescence microscopy images.

Statistical analysis

Statistical analyses were performed using PRISM 5.01 (GraphPad Software Inc.). Data are presented as mean $\pm$ standard deviation of *n* determinations as indicated in the figure legends. Animal survival was analyzed by log-rank tests, and *p*-values are as shown. All other data were analyzed by the Student's *t*-test, where *p* < 0.05 was considered significant. The Bonferroni correction was used for the multiple comparisons.

Results

Characteristics of hUCMSCs

After 5–10 days of culture, isolated cells started to grow as colonies of fibroblast-like cells (Fig. 1A1). Im-

munocytochemical detection of α -smooth muscle actin and vimentin confirmed the mesodermal origin of hUCMSCs (Fig. 1A2, A3). An analysis by flow cytometry revealed that these cells were negative for the expression of hematopoietic markers such as CD45, CD34, CD14, CD19, CD117, and also HLA-DR, but were positive for CD105, CD73, CD90, CD166, CD54, and CD13, which are generally considered for as markers of MSCs. The presence of major histocompatibility complex I on the membrane of hUCMSCs was consistent with the general description of MSCs. Affinity of hUCMSCs for extracellular matrix elements was suggested by a high expression of CD29 and CD44. Further, contamination of cultures with hematopoietic cells was excluded by more than 95% positive expression of CD105⁺CD45⁻, CD34⁻CD14⁻, and CD117⁻CD19⁻ (Table 1 and Supplementary Fig. S1).

The multiple differentiation potential of hUCMSCs was demonstrated by inducing osteogenic and adipogenic differentiation. Adipogenic differentiation was shown by the formation of intracytoplasmic lipid droplets that stained with Oil Red O (Fig. 1B). Osteogenic differentiation yielded an extracellular precipitate, which was identified as calcium deposits by the Alizarin Red stain (Fig. 1C). Additionally, they expressed genes either specific for adipocytes such as LPL, PPAR- γ , and adiponin (Fig. 1D) or specific for osteoblasts such as osteonectin, osteopontin, alkaline phosphatase (ALP), and collagen I (Fig. 1E).

FIG. 1. Characterization of hUCMSCs. (A) Morphology of hUCMSCs at day 10 of culture (A1) and immunocytochemical analysis of expanding hUCMSCs for α -smooth muscle actin (A2) and vimentin (A3). (B) Adipogenic differentiation of hUCMSCs was positively stained with Oil Red O. (C) Osteogenic differentiation of hUCMSCs was shown by calcium mineralization as revealed by Alizarin Red. (D) Adipogenic differentiation of hUCMSCs was further confirmed by expression of adipocyte-specific genes by RT-PCR. (E) Osteogenic differentiation of hUCMSCs was further confirmed by expression of bone-specific genes by RT-PCR. (A1) Bar = 150 μ m. (A2, A3, C, and D) Bar = 50 μ m. hUCMSCs, human umbilical cord matrix stem cells; RT-PCR, reverse transcriptase-polymerase chain reaction; PPAR- γ , peroxisome proliferator-activated receptor γ ; LPL, lipoprotein lipase; ALP, alkaline phosphatase. Color images available online at www.liebertonline.com/tea

TABLE 1. FLOW CYTOMETRY CHARACTERIZATION OF NATIVE HUMAN UMBILICAL CORD MATRIX STEM CELLS

Markers	hUCMSCs	Markers	hUCMSCs
CD105	99.15±0.75	CD19	2.70±1.27
CD90	99.67±0.32	CD14	3.01±1.48
CD73	99.06±0.61	HLA-ABC	46.73±11.26
CD44	99.12±0.77	HLA-DR	1.47±0.44
CD166	98.66±0.85	CD73 ⁺ CD90 ⁺	98.62±1.07
CD29	99.51±0.49	CD105 ⁺ CD45 ⁻	97.44±0.94
CD54	83.27±12.14	CD29 ⁺ CD44 ⁺	98.70±0.63
CD13	77.55±9.78	CD54 ⁺ CD13 ⁺	62.35±14.19
CD117	1.54±1.47	CD34 ⁻ CD14 ⁻	95.88±2.71
CD34	1.49±1.16	CD117 ⁻ CD19 ⁻	96.84±1.39
CD45	2.26±1.12	CD166 ⁺ HLA-DR ⁻	96.27±0.82

hUCMSCs, human umbilical cord matrix stem cells.

Expressing hepatic markers and functions of native hUCMSCs

We observed that undifferentiated hUCMSCs persistently expressed some hepatic markers and function both at the gene and protein levels. Phase I enzymes, such as cytochrome P450 (CYP) 1A1, CYP2C9, and tryptophan 2,3-dioxygenase (TDO), and key enzymes of urea cycle, such as carbamoyl phosphate synthetase-1 (CPS-1) and glutamine synthetase (GS), were expressed between passage 2 and passage 10 in native hUCMSCs, as determined by RT-PCR (Fig. 2A). In addition, synthetic proteins such as α -FP, ALB, and α 1-AT, as well as critical enzymes of glycometabolism (G6P), were expressed in hUCMSCs. On the other hand, gene expression of CYP2B6 and 3A4 isoforms and HNF-4 α and enzyme expression of tyrosine aminotransferase (TAT) were detected in AHHs, but not in native hUCMSCs (Fig. 2A). Analysis using immunocytochemistry further supported this observation. Immunocytochemistry revealed that most of the hUCMSCs stained positive for α -FP and ALB, and few expressed CK-18 and CK-19, whereas HepPar1 and HNF-4 α were not detected. Although the undifferentiated cells expressed ALB and CK-18, the expression level observed was much lower than that of AHHs (Fig. 2B).

We then examined the functional level of hUCMSCs. Periodic acid-Schiff (PAS) staining showed that native hUCMSCs were able to specifically store glycogen. Moreover, the stain was weaker than that detected in AHHs (Fig. 2C, D). ALB was secreted in the supernatants of native hUCMSC cultures, while the expression level was much lower than that observed for AHHs (Fig. 2E). Urea production was measured in cultures of native hUCMSCs exposed for 24 h to 0.5 mmol/L NH₄Cl and reached 10% of the level of AHHs under the same culture conditions (Fig. 2F).

In vitro differentiation of hUCMSCs into hepatocyte-like lineage

Morphological changes of hUCMSCs during differentiation were documented by phase-contrast microscopy. During the initiation step of hepatic differentiation, the cultured cells showed a remarkable transition from a bipolar fibroblast-like morphology (Fig. 3A1) to a round epithelial-like shape (Fig. 3A2). We can observe distinct small round or

oval-shaped cells with polyhedral structures, similar to those seen in primary AHHs (Fig. 3A3).

Immunocytochemical analysis of differentiated hUCMSCs showed persistence of ALB, CK-19, and CK-18. Notably, expression of the key CYP superfamily of oxidative metabolism enzymes, CYP7A1, was found to be activated, when the generated hepatocytes were at their most mature—further demonstrating increasing hepatic maturation in the differentiation cultures with time. Nevertheless, neither HepPar1 nor HNF-4 α was detected in differentiated cells (Fig. 3B).

Improve survival rate and inhibit liver enzyme release of ALF by direct transplantation of hUCMSCs

To compare the therapeutic potential of hUCMSCs with AHHs, 4×10⁷ hUCMSCs or AHHs/kg body weight were intrasplenically transplanted at 18 h after administration of CCl₄. Four-fifths of the animals treated with native hUCMSCs recovered from ALF, at the same time as 72.7% of the recipients were rescued by transplantation of AHHs at 28 days after transplantation. All animals in a negative control group died between 24 and 72 h after PBS treatment. A significant survival benefit was observed for both hUCMSC and AHH groups when compared with PBS treatment ($p < 0.05$), and there were no significant differences between hUCMSC and AHH groups ($p > 0.05$) (Fig. 4A).

As shown in Figures 4B and C, the serum AST and ALT levels in the injured mice increased to 4500 and 3700 IU, respectively, at 18 h after administration of CCl₄, confirming the infliction of acute extensive liver injury by CCl₄. The mean AST and ALT levels were dramatically decreased in the hUCMSC and AHH groups at 14 days after transplantation, and maintained at normal levels at 28 days. No significant differences of between the two groups were observed at any of the other time points.

hUCMSC therapy improves microscopic liver histopathology

To address our goal of whether native hUCMSCs reveal therapeutic abilities to regenerate an injured liver when compared with AHHs, hepatic necrosis and regeneration were evaluated in all groups by H&E staining. Pathological examination of H&E-stained sections revealed massive necrosis and hepatic lobule damage in the PBS group (Fig. 5A) compared with normal liver tissue (Fig. 5B), but hepatocyte necrosis was suppressed in both hUCMSC (Fig. 5C) and AHH groups (Fig. 5D) from day 14 after transplantation. Furthermore, the liver structure of the two groups recovered from abnormality at 28 days after transplantation (Fig. 5E, F).

Transplanted donor cells engraft in the mice and native hUCMSCs can differentiate into functional hepatocytes

To investigate the repopulation capacity of hUCMSCs or AHHs in the recipient liver, animals were killed 2 and 4 weeks after treatment, and the expression of hepatocyte-specific proteins was assessed by immunohistochemical detection of specific anti-human antibodies. These antibodies showed no crossreactivity for murine antigens. Transplantation of either hUCMSCs or AHHs resulted in predominantly periportal engraftment and expansion of human cells into the host mouse liver parenchyma. The early hepatic

FIG. 2. Expression of hepatic markers and functions by native hUCMSCs. **(A)** A representative the RT-PCR analysis of hUCMSCs from passage 2 (P2), P4, P6, P8, and P10 cultured in an expansion medium for late and early hepatic markers compared with AHHs. **(B)** Immunocytochemical analysis of expanding hUCMSCs and AHHs. **(C)** Native hUCMSCs displayed a weak positive reaction to PAS staining. **(D)** The efficiency of PAS staining was tested on AHHs. **(E)** ALB production of undifferentiated hUCMSCs and AHHs. **(F)** hUCMSCs were cultured in an expansion medium and were stimulated for 24 h with 0.5 mmol/L NH_4Cl before measuring the urea concentration in culture supernatants. Urea production was compared within cultures of AHHs. Data are means $\pm$ standard deviation; $n = 5$; $*p < 0.05$. Bar = 50 μm . AHHs, adult human hepatocytes; PAS, periodic acid-Schiff; CK, cytokeratin; CPS-1, carbamoyl phosphate synthetase-1; GS, glutamine synthetase; CYP, cytochrome P450; TAT, tyrosine aminotransferase; TDO, tryptophan 2,3-dioxygenase; α -FP, α -fetoprotein; ALB, albumin; HNF-4 α , hepatocyte nuclear factor 4 α . Color images available online at www.liebertonline.com/tea

FIG. 3. Effects of the hepatogenic differentiation protocol on hepatic specific proteins of hUCMSCs. **(A)** Morphological changes of undifferentiated hUCMSCs (**A1**) to differentiated hepatocyte-like cells (**A2**) under hepatogenic conditions. (**A3**) AHHs were used as a positive control. **(B)** Immunocytochemical analysis of differentiated hUCMSCs. **(A)** Bar = 100 μ m. **(B)** Bar = 50 μ m. Color images available online at www.liebertonline.com/tea

markers, α -FP, were found in small clusters of polygonal cells located near the portal veins at 2 weeks after hUCMSC transplantation, but were not detected after 4 weeks, indicating that those immature markers were downregulated *in vivo* (Fig. 6A1). Furthermore, CK-18, ALB, and HepPar1, the later hepatic markers, were detected on days 14 and 28 in host livers of either hUCMSC or AHH transplantation. These cells expressed ALB and HepPar1, which suggested that the hUCMSCs have differentiated into hepatocytes in the mouse liver (Fig. 6A2–A4). The positively stained hepatocytes were digitized to quantify the mature hepatocytes. The proportion of positively expressed ALB and HepPar1 did not exceed 1% in the livers of mice treated with hUCMSCs after 14 days and 28 days. Although the number of positive hepatocytes decreased gradually in the AHH group with time, the number was higher than those in the hUCMSC group at 14 days or 28 days post-transplantation (Fig. 6B, C).

To further show the functionality of transplanted cells, human ALB was quantified in the serum of rescued mice by ELISA. Normal SCID mouse serum was tested and showed

no crossreactivity with the ELISA reagents. Human ALB was found to be at a much higher concentration in the serum of mice transplanted with AHHs in comparison with levels in mice transplanted with hUCMSCs at 14 days or 28 days post-transplantation (Fig. 6D).

hUCMSC transplantation regulates systemic cytokine profiles

As reported previously, ALF induces a systemic inflammatory response. Analysis of mouse serum cytokine levels (Fig. 7) revealed a significant decrease in IL-1 β , TNF- α , and IL-6 after hUCMSC transplantation compared with PBS or AHH treatment. No significant differences of these proinflammatory cytokines, which are known to be upregulated during ALF, were observed between the PBS and AHH group. In contrast, levels of the anti-inflammatory cytokines IL-10 and IL-1RA were increased in hUCMSC-transplanted animals. A significant difference in the hUCMSC group was observed compared with the PBS or AHH group. Higher

FIG. 4. Direct transplantation of hUCMSCs or AHHs improves the survival rate and inhibits liver enzyme release of ALF. ALF was induced in NOD-SCID mice using 10% CCl₄ (0.28 mL/kg gavage). (A) Kaplan-Meier survival analysis of CCl₄ induced NOD-SCID mice. * $p < 0.05$ (log rank test). (B) AST and (C) ALT enzyme release levels in peripheral blood samples collected at pretransplantation, 14, and 28 days after transplantation. * $p < 0.05$. ALF, acute liver failure; NOD-SCID, nonobese diabetic severe combined immunodeficient; CCl₄, carbon tetrachloride; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PBS, phosphate-buffered saline.

levels of HGF were found for the hUCMSC and AHH groups when compared with the PBS group.

hUCMSC therapy enhances the recovery and proliferation of injured hepatocytes in vitro

Inhibition of hepatocellular death by hUCMSC therapy *in vivo* can either be a direct effect of trophic molecules

preserving liver cells or be an indirect effect, for example, via paracrine effects. Therefore, we tested the ability of hUCMSCs to indirectly inhibit the cell death of injured MHs by CCl₄. When hUCMSCs were cocultured indirectly with damaged MHs, a 46% increase in the fraction of viable cells was observed. With AHHs, no significant increase in the MH viability was seen (Fig. 8A). In parallel to these findings, the total amount of mouse ALB secreted was greatly increased in damaged MHs cocultured indirectly with hUCMSCs, but was not significantly altered in the presence of AHHs (Fig. 8B).

Hepatocyte replication is a major component of liver regeneration. To determine whether hUCMSCs can indirectly enhance hepatocyte replication, we explored the effect of coculturing with hUCMSCs on the *in vitro* proliferation of the damaged MHs. Proliferation of MHs was observed when these were cocultured with hUCMSCs or AHHs and visualized by double immunofluorescence staining for BrdU and ALB. With hUCMSC coculture, a 2.3-fold increase in BrdU-positive hepatocytes was observed. When damaged MHs were cocultured indirectly with AHHs, no significant increase in BrdU-positive hepatocytes was measured (Fig. 8C).

Discussion

MSCs derived from different tissues can generate hepatocyte-like cells *in vitro*, but not the fully functional, mature, and transplantable equivalents of hepatocytes that are isolated from an adult liver.⁶⁻⁸ The phenotypic profile of hUCMSCs isolated in our study corroborated results from previous studies with respect to expression of a consensus marker set of MSCs and a combination of several hepatic markers.²⁵ hUCMSCs submitted to the protocol of hepatic differentiation *in vitro* only reached a hepatocyte-like morphology, whereas mature hepatocyte markers were not detected. Moreover, a low level of hepatic function was found in undifferentiated hUCMSCs in comparison to AHHs. Thus, prehepatic differentiation of hUCMSCs *in vitro*, which also can increase the risk of microorganism contamination and chromosomal mutation, may be redundant. The previous study has demonstrated that direct transplantation of bone marrow-derived MSCs had a superior rescuing potential when compared to MSC-derived hepatocytes.¹⁷ Overall, we conclude that direct transplantation of native hUCMSCs may facilitate cell therapy of ALF rather than predifferentiation into hepatocyte-like cells.

It is important that during cell transplantation treatment, ALF inhibits hepatocyte necrosis and ameliorates the liver function, thereby reducing the mortality. In our study, it was found that intrasplenic transplantation of as few as 4×10^7 hUCMSCs or AHHs/kg body weight (approximating 2×10^9 cells for an average adult body weight of 50 kg) effectively rescued 80% of the recipient mice from ALF. The cell doses administered are comparable to those currently used for hepatocyte transplantation into patients with ALF.³¹ The recipient mortality was apparently reduced by hUCMSC or AHH treatment compared with the PBS control group. Furthermore, the concentrations of AST and ALT, which are two important indicators of liver injury, were significantly decreased after hUCMSC or AHH transplantation. Histological evaluation of liver tissue after

FIG. 5. Histopathological recovery of CCl₄-induced ALF transplanted with hUCMSCs or AHHs. Hematoxylin and eosin staining showed that there were massive necrosis and hepatic lobule damage in the PBS group (A) compared with normal liver tissue (B). In contrast, (C, D) hepatocyte necrosis was suppressed in both hUCMSC and AHH groups from day 14 after transplantation. (E, F) the liver structure of the two groups recovered from abnormality at 28 days after transplantation. Bar=50 μ m. Color images available online at www.liebertonline.com/tea

cell treatment provided initial insights into the cellular targets of therapy. A striking recovery of liver structure was seen after transplantation, suggesting that hUCMSCs may reconstitute liver tissue in a similar manner to AHHs by either differentiating into hepatocytes or promoting endogenous host regeneration. These results confirmed that the direct transplantation of native hUCMSCs can rescue ALF and repopulate livers of mice as an efficient alternative source of AHHs. Transplantation of MSCs derived from other tissues has recently shown similar results. However, the different mechanisms of actions that occur between hUCMSC- and AHH-mediated liver therapy remains uncertain.

ALF is typically associated with massive hepatocellular necrosis.³² Consequently, transplanted hepatocytes or differentiation into mature hepatocytes compensated the cellular losses should play an important role for the cell therapy associated with ALF.³¹ In the AHH group, more than 10% of all hepatocytes in the host liver were replaced by human hepatocytes after 4 weeks, indicating that transplanted AHHs can compensate for the cell loss. It is generally accepted that a repopulation of 1–5% of the he-

patic mass, which can be easily achieved in the AHHs group, is sufficient to correct liver disorders in most injury models.³¹ Although the proportion of hepatocytes derived from hUCMSCs was <1% of the total hepatocytes, it can be concluded from this percentage that hUCMSCs can differentiate into hepatocytes *in vivo* and may play some extent compensatory roles for the recovery of liver function. The functional compensation was further confirmed by detecting quantities of human ALB in the recipient mouse serum. The number of hepatocytes derived from transplanted hUCMSCs in the recipient mice livers was less than the frequency of HepPar1- and ALB-positive cells in the AHH-transplanted mouse livers. Meanwhile, we also observed that hUCMSCs produced significantly less human ALB than AHHs in the recipient mice. Together with the data suggesting a similar rescuing potential that exists between hUCMSCs and AHHs, these findings suggest that the differentiation of hUCMSCs into hepatocytes was not primarily attributed to ALF.

Stimulation of endogenous regeneration represents another potential mechanism of an MSC-based therapeutic effect, previously described in models of myocardial

FIG. 6. Native hUCMSC- or AHH-engrafted livers of CCl₄-induced ALF in NOD-SCID mice. **(A)** Human UCMSCs or AHHs were injected into the spleen of ALF and were tracked into liver recipients 2 and 4 weeks later by immunostaining with a specific human antibody. **(B)** Quantification of human ALB-positive cells. **(C)** Quantification of human HepPar1-positive cells. **(D)** Human ALB was determined in the serum of transplanted mice by ELISA specifically detecting human ALB. * $p < 0.05$. Bar = 50 μ m. ELISA, enzyme-linked immunosorbent assay; FOV, field of view. Color images available online at www.liebertonline.com/tea

infarction.³³ We have shown that hUCMSC transplantation was even more effective than treatment with AHHs and successfully downregulated the systemic inflammation, as determined by measuring serum levels of IL-1 β , TNF- α , IL-6, IL-10, and IL-1RA. Prostaglandin E2 (PGE2)³⁴ and human leukocyte antigen-G5 (HLA-G5)¹³ were produced by hUCMSCs. PGE2 promotes the expression of the anti-inflammatory cytokine IL-10 and inhibits their expression of proinflammatory cytokines such as TNF- α and IL-1 β .³⁵ HLA-G5 is the soluble counterpart of membrane-bound HLA-G1 and can be secreted by MSCs. There is an important positive feedback loop between HLA-G5 and IL-10.³⁶ MSCs secrete high levels of IL-1RA, which can block the proinflammatory cytokines of IL-1.³⁷ IL-6 is produced during the acute phase of the inflammatory reaction³⁵ and elevated in patients with

failure liver.³⁸ It is reported that hUCMSCs have an immunosuppressive potential ability to suppress the expression of the proinflammatory cytokine IL-6.³⁹ The upregulation of HGF indicated that liver regeneration was enhanced. Furthermore, *in vitro* studies demonstrated that coculture indirectly with hUCMSCs can promote the viable functional recovery and facilitate cell replication of the damaged MHs induced by CCl₄ rather than AHHs, suggesting that paracrine effects play a key role in the rescue of damaged hepatocytes. This correlated with observations in previous studies in CCl₄-induced liver injury that hUCMSC therapy inhibited the hepatocellular apoptosis and stimulated liver regeneration *in vivo*.⁴⁰ It has been known that MSCs synthesize a wide variety of growth factors and cytokines, exerting a paracrine effect on local cellular dynamics.⁴¹ Such trophic effects are

FIG. 7. Cell transplantation alters the systemic cytokine profile in the serum of CCl_4 -induced ALF. Serum samples were collected from mice 36 h after PBS, hUCMSC, or AHH transplantation. Concentration of IL-1 β (A), TNF- α (B), IL-6 (C), IL-10 (D), HGF (E), and IL-1RA (F) was measured by ELISA. * $p < 0.05$. IL, interleukin; TNF- α , tumor necrosis factor- α ; HGF, hepatocyte growth factor; IL-1RA, IL-1 receptor antagonist.

also believed to enhance endogenous liver regeneration. The contribution of paracrine effects is further supported by a recent study reporting the therapeutic benefit of using MSCs-derived biomolecules for the treatment of acute liver injury.⁴⁰ AHHs have produced less stimulatory factors and more molecules primarily associated with liver functions. Therefore, the primary effect of hUCMSCs rescuing ALF may be attributed to the stimulation of endogenous liver regeneration through paracrine effects. Compensated liver function is the sole effect of AHHs. Further investigations are needed to determine the type of molecules derived from hUCMSCs that lead to the endogenous liver regeneration during the rescue of AFL mice.

In summary, the current study shows that the native hUCMSC therapy has a similar ability to rescue ALF as that observed for AHHs. We further demonstrate that hUCMSCs provide paracrine effects on stimulating endogenous liver

regeneration rather than hepatic differentiation for compensated liver function during the cell therapy of ALF. This work validates that direct transplantation of hUCMSCs could be an alternative treatment as hepatocyte transplantation for cell therapy of ALF, and therefore eliminate the shortage of hepatocytes.

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FIG. 8. Indirect coculture with hUCMSCs enhances the recovery and proliferation of the damaged MHs by CCl_4 inducing *in vitro*. (A) The viability of the damaged MHs cocultured with hUCMSCs. (B) ALB secretion of MHs cocultured with hUCMSCs. (C) Quantification of BrdU-positive hepatocytes by an image analysis. * $p < 0.05$. MHs, mouse hepatocytes.

Disclosure Statement

The authors indicate no potential conflicts of interest.

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