Transplantation of Human Umbilical Cord Blood Mesenchymal Stem Cells Improves Survival Rates in a Rat Model of Acute Hepatic Necrosis

Ling-Ling Shi, MD, Fu-Ping Liu, MS and De-Wen Wang, MS

Abstract: Introduction: Stem cell-based therapies are emerging as important and promising methods in the treatment of end-stage liver disease. This study is aimed to evaluate the effects of human umbilical cord blood mesenchymal stem cell (HUCBMSC) transplantation in acute hepatic necrosis (AHN). Methods: Green fluorescent protein (GFP)-labeled HUCBMSCs were injected into the liver of rats in which AHN was induced by carbon tetrachloride, and the migration of these cells in liver slices was evaluated from 48 hours to 4 weeks post-transplantation. The transdifferentiation status of the HUCBMSCs was evaluated using immunohistochemistry and real-time reverse transcription-polymerase chain reaction, and survival rates were statistically analyzed. Results: Dispersed GFP fluorescence was observed along the portal area 48 hours after transplantation. One week post-transplantation, GFP-positive cells were found in necrotic liver areas, and GFP-positive cells persisted after 4 weeks. Immunohistochemistry and real-time polymerase chain reaction analysis showed that transplanted HUCBMSCs expressed several human liver tissue-specific markers in rats with AHN. Statistical analysis revealed that rats receiving no HUCBMSCs had significantly lower death rates after 48 hours than those receiving no HUCBMSCs. Conclusion: HUCBMSC transplantation can significantly improve the survival of rats with AHN. The underlying mechanisms involved may include the transdifferentiation of HUCBMSCs into hepatocyte-like cells and targeted migration of these cells to liver lesion sites.

Key Indexing Terms: Human umbilical cord blood mesenchymal stem cells; Acute hepatic necrosis; Survival rates; Transdifferentiation; Hepatocyte-like cells; Targeted migration. [Am J Med Sci 2011;342(3):212-217.]

Liver transplantation is an effective method for the treatment of acute severe hepatitis or end-stage liver disease. However, multiple factors, such as donor scarcity, surgical risk and expense and potential immunological rejection, limit the application of transplantation. Stem cell-based therapy could offer an alternative to liver transplants, and stem cell transplantation has reportedly already been used to treat end-stage liver disease. As mesenchymal stem cells (MSCs) are capable of transdifferentiating into hepatic-like cells, autologous cell transplantation of MSCs could be a convenient and feasible treatment for end-stage liver diseases.

Recent in vitro studies showed that bone marrow-derived mesenchymal stem cells (BMMSCs) can transdifferentiate into hepatocyte-like cells. Furthermore, an in vivo study showed that the transplantation of BMMSCs into fumarate acetyl acid hydroxylase gene defect mice improved liver function, and that the BMMSC-derived cells possessed the function of mature liver cells. In addition, when purified human and rat BMMSCs were introduced into the liver during biliary stasis via the portal vein, these cells went on to exhibit organelle ultrastructure reminiscent of mature hepatocytes and expressed liver cell specific markers such as albumin (ALB), alpha-fetoprotein (AFP) and cytokeratin 18 (CK18). In addition, transplanted adipose-derived stem cells can differentiate into hepatocyte-like cells, resulting in the improvement of liver function. A previous study describing the effects of BMMSCs on the outcome of patients with end-stage liver disease was evaluated preclinically, and it was suggested that BMMSCs could significantly improve the score of end-stage liver disease. Similar to BMMSCs, human umbilical cord blood mesenchymal stem cells (HUCBMSCs) are pluripotent. In fact, HUCBMSCs have recently been considered an alternative source of MSCs. However, reports on the application of HUCBMSCs in the treatment of liver diseases are still relatively rare.

To better understand the effects of stem cell transplantation in end-stage liver disease, we generated enhanced green fluorescent protein (EGFP)-labeled HUCBMSCs (EGFP-HUCBMSCs) via transfection of HUCBMSCs with EGFP and investigated the effect of transplanted EGFP-HUCBMSCs on the outcome of carbon tetrachloride (CCL4)-induced acute hepatic necrosis (AHN) in rats.

MATERIALS AND METHODS

Isolation, Culture and Identification of HUCBMSCs

Cord blood of healthy pregnant mothers (70–100 mL) was collected after signed informed consent was obtained. After dilution with saline medium (1:1 ratio), the diluted cord blood was placed on lymphocyte separation medium (Ficoll, 1.077 g/mL) and centrifuged. The mononuclear cell-enriched buffy layer was collected. After cell counts and concentration adjustments, cells were cultured with human MSC Basal Medium (Mesencult; StemCell Technologies Inc., Vancouver, Canada) at a density of 1 × 10^5/cm^2 at 37°C in 5% CO2 with saturated humidity. Culture medium was replaced after 3 days, and then every 4 days afterward, or when the color of medium changed to yellow. After passing 3 times, cells were digested with 0.25% trypsin at 90% confluence. The detached cells were incubated with phycoerythrin (PE)-conjugated rabbit antiserum of differentiation PE-CD29 (Becton Dickinson [BD], San Diego, CA), PE-CD44 (BD), PECD105 (BD), fluorescein isothiocyanate (FITC)-conjugated antibodies FITC-CD45 (BD) and FITC-CD34 (BD), respectively, and subjected to a cytometric assay.

Preparation of EGFP-HUCBMSCs

HUCBMSCs were adjusted to a concentration of 1 × 10^6/mL and seeded into 6-well plates. When the cell cultures...
reached 70% confluence, polybrene-facilitated transfection was performed using a viral vector encoding EGFP (70 μL vector, 0.3 μL polybrene/well; Cyagen Biosciences, Guangzhou, China). The culture medium was replaced after 10 hours. After 2 additional days of cell culture, the medium was replaced with fresh medium containing neomycin (600 μg/mL; Sigma, St. Louis, MO), and the selection pressure was maintained until most of the untransfected cells in the control cultures died.

**Rat Model of AHN and HUCBMSC Treatment**

Eighty specific pathogen-free grade Sprague Dawley rats (weight, 160–200 g; 40 males and 40 females) were raised in Guangdong Medical Experimental Animal Center. Rats were divided into 4 groups (20 rats each group), and each group received different pretreatments: (1) in the group of AHN model rats with HUBMSC treatment, 3 × 10^6 to 5 × 10^6 EGFP-HUBMSCs were first directly injected into the liver at multiple sites via laparotomy and subsequently, 50% CCl_4 (3 mL/kg) in olive oil solution was injected intraperitoneally 24 hours later; (2) in the group of AHN model control in which AHN model rats received mock treatment, saline solution (3 mL/kg) was first directly injected into the liver at multiple sites via laparotomy and subsequently, 50% CCl_4 (3 mL/kg) in olive oil solution was injected intraperitoneally 24 hours later; (3) in the group of normal control, rats were left untreated and (4) in the group of normal rat treated with HUBMSC, which served for comparative analysis on the specific effect of HUBMSC on AHN, 3 × 10^6 to 5 × 10^6 EGFP-HUBMSCs were directly injected into the liver at multiple sites via laparotomy without subsequent CCl_4 treatment. All animal experiments in this study complied with ethical requirements and were approved by the local ethics committee.

**Fluorescent Microscopy Analysis**

Four rats from each group were killed 24 hours, 48 hours, 72 hours, and 1 week after transplantation; and 2 rats from each group were killed 2 and 4 weeks after transplantation. Livers were removed, and frozen sections of liver tissue were prepared and analyzed via fluorescence microscopy (Olympus, Japan).

**Immunohistochemistry**

Livers were removed from rats 1 week after transplantation, fixed with formalin, embedded in paraffin, cut into 3-μm sections and mounted onto slides for immunohistochemistry analysis. Briefly, the slides were incubated with anti-ALB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-AFP (Santa Cruz Biotechnology, Inc.) or anti-Hep Par 1 antibodies ( Dako, Glostrup, Denmark) at 37°C for 120 minutes. After washing with phosphate-buffered saline for 3 minutes, slides were further incubated with a horseradish peroxidase-conjugated secondary antibody at 37°C for 20 minutes. After 3 washes with phosphate-buffered saline, the slides were developed using diaminobenzidine as substrate for 2 to 5 minutes, dehydrated, mounted with coverslips, sealed with rubber cement and analyzed via microscopy.

**Serology Assay**

The sera of HUCBMSC-transplanted rats and control rats were collected at various time points (24 hours, 72 hours and 1 week) and subjected to a serum test of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels using a CTRL kit (Shanghai Fosun Long March Medicine Science Co, Ltd, Shanghai, China).

**Reverse Transcription and Real-Time Polymerase Chain Reaction**

One week after transplantation, rat livers were removed, and total RNA was extracted from the liver tissue. cDNA was synthesized from 1 μg of RNA as template using Moloney Murine Leukemia Virus reverse transcriptase (MBI) and random primers. The primer sets specific to ALB, AFP and Hep Par 1 were designed by GenBank sequences and were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA; Table 1). The polymerase chain reaction (PCR) was performed as follows: denaturing at 93°C for 3 minutes; amplification for 40 cycles at 93°C for 30 seconds, 55°C for 45 seconds and 72°C for 45 seconds; followed by an extension at 72°C for 10 minutes. PCRs were performed in triplicate. The relative abundance of target genes was calculated using LightCycler Software 4.05 (Roche Diagnostics, Indianapolis, IN). β-Actin was used as the internal standard.

**Statistical Analysis**

The survival rate of each group was compared using χ^2 tests. Differences between groups were analyzed using an independent samples t test, and P < 0.05 was considered statistically significant.

**RESULTS**

**Preparation of HUCBMSCs and EGFP-HUCBMSCs**

Microscopic analysis showed that cultured HUCBMSCs had a fibroblast-like shape and were arranged in a spiral pattern (Figure 1A). In addition, flow cytometry analysis showed that HUCBMSCs had high positive rates for CD29, CD44 and CD105 expression (97.87%, 98.44% and 84.40%, respectively) and low positive rates for CD34 and CD45 expression (12.76% and 9.64%, respectively). Furthermore, transfected EGFP-HUCBMSCs exhibited bright green fluorescence (Figure 1B).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size (bp)</th>
<th>Primer sets</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-AFP</td>
<td>74</td>
<td>Forward primer</td>
<td>5'-GGA GCG GCT GAC ATT ATT ATC G-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer</td>
<td>5'-TGG CCA ACA CCA GGG TTG TTT A-3'</td>
</tr>
<tr>
<td>H-ALB</td>
<td>68</td>
<td>Forward primer</td>
<td>5'-TCT TAC CAA AGT CCA CAC GGA AT-3'</td>
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<tr>
<td></td>
<td></td>
<td>Reverse primer</td>
<td>5'-GTT CCC CCC TGG CAT CAG-3'</td>
</tr>
<tr>
<td>H-CK18</td>
<td>68</td>
<td>Forward primer</td>
<td>5'-TGG CGA GGA CTT TAA TCT TGG T-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer</td>
<td>5'-TGG TCT TTT GGA TGG TTT GCA-3'</td>
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<tr>
<td>H-β-actin</td>
<td>106</td>
<td>Forward primer</td>
<td>5'-GCA TGG GTG AGA AGG ATT CTC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse primer</td>
<td>5'-TGG TCC CAG TTG GTG AGC AT-3'</td>
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</table>
Serological Markers

We assayed the levels of ALT and AST in serum from normal control, AHN model control and HUCBMSC-transplanted AHN rats and found that (1) the levels of ALT and AST in AHN model control were significantly higher than those in normal control rats at 3 time points, implicating the successful establishment of the AHN model and (2) the levels of ALT and AST in HUCBMSC-transplanted rats were lower than those in AHN model control at 3 time points, and the differences at 72 hours were statistically significant (Table 2). This suggests that liver inflammation and necrosis were ameliorated in HUCBMSC-transplanted rats compared with AHN controls.

Survival Rates

The survival rates of experimental and control rats were determined 72 hours after transplantation. Normal control rats had a survival rate of 100%, whereas normal rats treated with EGFP-HUCBMSCs alone had a survival rate of 95%. Of AHN model rats, 12 rats died within 24 hours, 2 died between 24 and 48 hours and the remaining 8 died between 48 and 72 hours. However, in HUCBMSC-transplanted AHN rats, 6 rats died within 24 hours, but the remaining 14 rats survived to 72 hours (Table 3). Statistical analysis showed that CCl4-treated rats had a survival rate of 100%, whereas normal rats treated with EGFP-HUCBMSCs alone (Figures 3D–F).

Distribution of Transplanted EGFP-HUCBMSCs in the Liver

The migration of EGFP-HUCBMSCs in the liver was traced via fluorescent microscopy. In HUCBMSC-transplanted AHN rats, the fluorescent signal had dispersed from the injection site to the periportal area 24 hours after transplantation (Figure 2A). EGFP fluorescence could also be observed 48 hours after transplantation in necrotic lesions (Figure 2B), and EGFP-positive cells were still visible 4 weeks post-transplantation (Figure 2C). In normal rats treated with EGFP-HUCBMSCs, EGFP-positive cells could be seen around the periportal area 48 hours after transplantation (Figure 2D), but no EGFP-positive cell dispersal was found (Figure 2E). EGFP fluorescence faded significantly by 4 weeks post-transplantation (Figure 2F).

Immunohistochemistry

AFP, ALB and Hep Par I-positive cells were visible in the liver sections of HUCBMSC-transplanted AHN rats. Specifically, the human-specific marker Hep Par I was found in 90% of liver slices, with the staining intensity peaking 1 week post-transplantation (Figure 3A). ALB staining was detected 1 to 2 weeks post-transplantation (Figure 3B), but the number of ALB-positive cells was fewer than that of Hep Par I. Approximately 50% of liver slices were positive for AFP staining, and the number of positive cells increased between 24 hours and 1 week post-transplantation, with the number of positive cells slightly decreasing thereafter (Figure 3C). However, no positive staining for Hep Par I, AFP or ALB was found in the livers of normal control rats or normal rats treated with EGFP-HUCBMSCs alone (Figures 3D–F).

TABLE 2. ALT and AST levels in AHN rat serum

<table>
<thead>
<tr>
<th></th>
<th>ALT (IU/mL)</th>
<th>AST (IU/mL)</th>
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<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
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<tr>
<td>AHN</td>
<td>198.45 ± 15.02a</td>
<td>154.27 ± 11.82a</td>
</tr>
<tr>
<td>AHN + HUCBMSCs</td>
<td>130.00 ± 2.26</td>
<td>92.55 ± 1.48b</td>
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<tr>
<td>Normal rats</td>
<td>39.15 ± 6.718</td>
<td>40.95 ± 5.02</td>
</tr>
</tbody>
</table>

a AHN control vs. normal rats, P < 0.05.
b AHN + HUCBMSCs vs. AHN, 72-h post-transplantation, P < 0.05.

TABLE 3. Survival rates of AHN rats with or without HUCBMSCs transplantation

<table>
<thead>
<tr>
<th>Groups</th>
<th>n (24 h)</th>
<th>% (24 h)</th>
<th>n (48 h)</th>
<th>% (48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHN rats + HUCBMSCs</td>
<td>20</td>
<td>14</td>
<td>70a</td>
<td>14</td>
</tr>
<tr>
<td>AHN rats</td>
<td>20</td>
<td>8</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>Normal rats + HUCBMSCs</td>
<td>20</td>
<td>19</td>
<td>95</td>
<td>19</td>
</tr>
<tr>
<td>Normal rats</td>
<td>20</td>
<td>20</td>
<td>100</td>
<td>20</td>
</tr>
</tbody>
</table>

a χ² test: χ² = 3.64, P = 0.057 > 0.05 vs. AHN rats.
b χ² test: χ² = 15.00, P = 0.000 < 0.01 vs. AHN rats.
Quantitative PCR Analysis of AFP, ALB and CK18

The relative levels of AFP, ALB and CK18 mRNAs present in rat livers were determined using quantitative PCR. Although the expression levels of AFP, ALB and CK18 in EGFP-HUCBMSC-transplanted AHN rats were higher than in AHN model rats that did not receive EGFP-HUCBMSC transplants, only CK18 expression levels were significantly different between the 2 groups (Table 4; \( P < 0.05 \)).

FIGURE 2. Migration of HUCBMSCs. (A) Twenty-four hours after transplantation, transplanted HUCBMSCs were distributed in the periportal area of CCl\(_4\)-treated rats. (B) One week after transplantation, transplanted HUCBMSCs were observed in the necrotic lesion area in CCl\(_4\)-treated rats. (C) Four weeks after transplantation, transplanted HUCBMSCs had dispersed into the area of liver necrosis in CCl\(_4\)-treated rats. (D) Forty-eight hours after transplantation, GFP fluorescence was observed around the periportal area in normal rats treated with EGFP-HUCBMSCs. (E) One week after transplantation into normal rats, no migration of EGFP-HUCBMSCs from the periportal area into the necrotic lesion area was observed, and GFP fluorescence appeared weak. (F) Four weeks post-transplantation, GFP expression was significantly reduced in normal rats transplanted with EGFP-HUCBMSCs.

FIGURE 3. Immunohistochemical analysis. One week after transplantation of HUCBMSCs into CCl\(_4\)-treated rats, liver sections were analyzed via immunohistochemistry. (A) Hep Par I staining, (B) AFP staining, (C) ALB staining and (D–F) Hep Par I, AFP and ALB staining in liver sections of control animals, respectively.
DISCUSSION

In this study, we evaluated the effects of transplanted GFP-labeled HUCBMSCs on the survival rates of rats with CCl4-induced AHN. We found that the survival rates of CCl4-treated rats receiving a transplant of HUCBMSCs were significantly higher than those of rats treated with CCl4 alone, suggesting HUCBMSCs were effective in the treatment of AHN. This result is consistent with previous reports on the effectiveness of lineage-committed stem cells in the treatment of liver disease. For example, transplantation of BMSCs, adult stem cells and embryonic stem cells has shown beneficial effects in the treatment of liver injury or disease.13–15

To probe the underlying mechanism for the beneficial effects of EGFP-HUCBMSCs in AHN, we traced the migration of transplanted cells in liver sections and found that transplanted EGFP-HUCBMSCs migrated to the portal area and target lesion sites. This scenario is reminiscent of a previous report showing the targeted migration of transplanted BMSCs to liver lesion sites.16 Although the molecular mechanism underlying targeted migration of HUCBMSCs is not clear in this study, previous studies suggest the involvement of cytokines such as hepatocyte growth factor (HGF), CXCR4, stromal-derived factor 1 and c-Met in the targeted migration of BMSC/CBMSCs after transplant.17 As the upregulation of hepatocyte growth factor and stromal-derived factor 1 after liver injury is a frequent event, we speculate that the targeted migration of EGFP-HUCBMSCs may use pathways similar to BMSC/CBMSCs.

Our data suggest that HUCBMSCs are competent to differentiate into functional hepatic cells. First, the timing profile of Hep Par 1 expression we observed, which began within 24 hours and peaked 1 week after transplantation, may represent the timeline for hepatocyte differentiation of transplanted HUCBMSCs. In addition, the expression timing profiles of AFP (relatively early) and ALB (relatively late) seem consistent with the general timing of hepatocyte development. Furthermore, the increase in levels of expression of the human liver-specific marker CK18 also supports the assertion that transplanted HUCBMSCs are capable of hepatocyte differentiation. Although the levels of AFP and ALB mRNA were not significantly different between experimental animals and controls, this inconsistency is likely due to the relatively small sample size used in this study. Nevertheless, the detailed ontology of the transplanted HUCBMSCs is not fully clear. The possibility that the phenotypes of differentiated HUCBMSCs could derive from fusion with host liver cells cannot be excluded. When hematopoietic stem cells are transplanted into animal models of liver injury, cells possessing a hybrid phenotype of the host liver cell and the transplanted hematopoietic stem cells came about as a result of fusion between donor and recipient cells.18–20

The timing of HUCBMSC differentiation after transplantation in our study suggests that the improved survival rates we observed at the early time points are not due to mature hepatocytes or hepatic-like cells differentiated from HUCBMSCs. Based on this observation, there may be a functional role for undifferentiated HUCBMSCs in this context. These results are consistent with previous work with MSCs/BMMSCs, which showed that MSCs can act through a paracrine signaling mechanism in the treatment of liver diseases,21,22 and that undifferentiated BMSCs are more competent to suppress liver fibrosis than hepatic-like cells differentiated from BMSCs.15

In conclusion, our study showed that transplantation of HUCBMSCs significantly improves survival rates in a rat model of AHN. The underlying mechanisms involved may include the transdifferentiation of HUCBMSCs into hepatocyte-like cells and targeted migration of these cells to liver lesion sites.

REFERENCES


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**TABLE 4. The mRNA level of AFP, ALB and CK18 1 week after HUCBMSCs transplantation**

<table>
<thead>
<tr>
<th>Group</th>
<th>AFP</th>
<th>ALB</th>
<th>CK18</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHN rats + HUCBMSCs</td>
<td>140.00 ± 51.96</td>
<td>4.63 ± 4.72</td>
<td>4377.00 ± 1504.44</td>
</tr>
<tr>
<td>AHN rats</td>
<td>37.33 ± 43.88</td>
<td>1.67 ± 0.81</td>
<td>11.47 ± 16.16</td>
</tr>
</tbody>
</table>

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" AHN rats + HUCBMSCs vs. AHN rats, P = 0.166 > 0.05.
" AHN rats + HUCBMSCs vs. AHN rats, P = 0.428 > 0.05.
" AHN rats + HUCBMSCs vs. AHN rats, P = 0.032 < 0.05.


