Effect of allogeneic umbilical cord mesenchymal stem cell transplantation in a rat model of hepatic cirrhosis

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Abstract

OBJECTIVE: To determine the effects of human umbilical cord mesenchymal stem cell (UCMSC) transplantation, alone or in combination with tanshinone IIA (Tan IIA) on hepatic cirrhosis in rats.

METHODS: A rat model of cirrhosis was established. Rats were divided into control, UCMSC, and UCMSC plus Tan IIA groups. Rats in the UCMSC group were injected via the tail vein with 0.2 mL DiI-labeled UCMSC suspension. Intraperitoneal Tan IIA injections (20 mg/kg) were started on the day of UCMSC transplantation in the UCMSC plus Tan IIA group, and continued for 7 consecutive days thereafter. Rats were sacrificed 1 day, 3 days, 1 month, and 3 months after transplantation and the numbers of DiI-labeled UCMSCs colonizing the liver were determined. Albumin (ALB) and alanine aminotransferase (ALT) levels were measured in venous blood, and mRNA and protein expression levels of human ALB and cytokeratin (CK)-18 in liver tissues were determined by reverse transcription-polymerase chain reaction and western blotting, respectively.

RESULTS: Serum ALT levels were significantly lower and serum ALB levels significantly higher in rats in the UCMSC group compared with the control group (P < 0.05). Hepatic CK-18 and ALB mRNA and protein expression levels increased after transplantation, and were significantly higher in the UCMSC plus Tan IIA group compared with the UCMSC group (P < 0.05).

CONCLUSION: Human UCMSCs transplanted into rats with liver cirrhosis can grow and differentiate into hepatocyte-like cells resulting in improved liver function in vivo. Tan IIA further influenced transplantation outcomes.

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Key words: Liver cirrhosis; Umbilical cord; Mesenchymal stem cells; Tanshinone; Transplantation

INTRODUCTION

Cirrhosis is a major human health problem that consumes social resources. Treatments for liver cirrhosis...
have been extensively researched, and liver transplantation has been shown to prolong the survival of patients with cirrhosis. However, transplantation is expensive, donors are limited, and the procedure is associated with ethical issues, all of which seriously limit its clinical application. Biological artificial liver treatment cannot resolve the underlying pathological process, which fundamentally decreases the number of functional liver cells. However, stem cell research may offer a novel treatment for liver cirrhosis. Various stem cells have been shown to be useful for liver cell regeneration and repair. Although most studies have investigated the application of bone marrow mesenchymal stem cells (MSCs), few human bone marrow MSCs differentiate into liver cells. In addition, the acquisition of bone marrow MSCs increases the morbidity of patients with liver cirrhosis. Umbilical cord MSCs (UCMSCs) are capable of multi-directional differentiation, and thus have potential for the treatment of liver cirrhosis. UCMSCs exhibit the general characteristics of stem cells: they have an original phenotype without spontaneous differentiation, do not express graft-versus-host disease-associated antigens, possess similar morphology and immunophenotype, but show lower proliferation and differentiation abilities than bone marrow MSCs.

Tanshinone IIA (Tan IIA) is a natural antioxidant derived from Danshen (Radix Salviae Miltiorrhiza). It has been shown to relieve pain and promote blood circulation. Tan IIA is a natural antioxidant with hepatoprotective, anti-fibrosis, and anti-cancer effects, and has also shown potential for inducing stem cell proliferation and differentiation. In this study, we investigated the effects of UCMSC transplantation, alone and in combination with Tan IIA, on serum levels of albumin (ALB) and alanine aminotransferase (ALT), and liver human cytokeratin (CK)-18 and ALB mRNA and protein levels in a rat model of liver cirrhosis.

**MATERIALS AND METHODS**

**Establishment of rat model of liver cirrhosis**
Sixty healthy female specific pathogen-free grade Sprague-Dawley rats [(280±20) g; 6-8 weeks old] were obtained from the Experimental Animal Center of Yangzhou University. The rats were randomly divided into the following groups: control group (10 rats), intraperitoneal injection of physiological saline, 0.75 mL/kg; and experimental group (50 rats), intraperitoneal injection of 15% carbon tetrachloride 22 in liquid paraffin oil, 0.75 mL/kg, three times per week for 8 weeks. All rats were allowed free access to drinking water and food. After 8 weeks, four rats were randomly selected from each group and examined for pathological changes indicative of cirrhosis of the liver.

**Human UCMSC transplantation**
The cirrhotic rats were divided into three groups: control group (15 rats); UCMSC group (15 rats); and US-CMC plus Tan IIA group (15 rats). In the UCMSC group, 0.2 mL of a suspension containing approximately 2 × 10^6 Dil-labeled UCMSCs was injected into the tail vein. In the UCMSC plus Tan IIA group, intraperitoneal injection of Tan IIA (20 mg/kg) injections were started on the same day as UCMSC transplantation and continued for 7 consecutive days thereafter. Rats were sacrificed 1 day, 3 days, 1 month, and 3 months after transplantation to measure ALB and ALT levels in venous blood.

**Flow cytometry for surface marker identification**
Following enzymatic digestion, passage-3 cells (1 × 10^6 cells/mL) were treated with TrypLE (Invitrogen, No. 12604021), and then washed twice with phosphate-buffered saline (PBS). Aliquots of cells were transferred to test tubes and incubated with CD14-FITC (Beckman Coulter), CD45-PE (Beckman Coulter), CD79a-allophycocyanin (APC) (Abcam), CD90-APC (R&D), CD34-phycoerythrin (PE) (Abcam, ab46925), CD73-PE (Santa Cruz), CD105-PE (eBioscience), and human leucocyte antigen (HLA)-DR-PE (eBioscience) antibodies at 4 ℃ for 30 min in the dark. Surface markers were detected using flow cytometry after washing twice with PBS. FITC-, PE-, and APC-labeled mouse immunoglobulin isotypes, respectively, were used as negative controls.

**Detection of ALB and CK-18 mRNA in liver tissue**
Total RNA was extracted from frozen liver tissue using Trizol, and used for cDNA synthesis by reverse transcription (RT). The cDNA was then used as a template for polymerase chain reaction (PCR) amplification according to the following protocol: degeneration at 94 ℃ for 30 s, annealing at 94 ℃ for 5 s, and extension at 55 ℃ for 30 s, for 45 cycles. The primer sequences are given in Table 1.
Detection of ALB and CK-18 proteins in liver tissue
Liver tissue 500-600 mg and 1 mL lysed tissue extract were centrifuged (15000 × g) and the supernatant was stored at -80 °C for Bradford protein assay. We prepared a separation gel (8%) and a stacking gel (4.5%). The first sample was separated using vertical electrophoresis at a voltage of 80 V for 30 min, and then electrophoresed at 110 V for 60 min. The bands were then electrotransferred to a polyvinylidene fluoride membrane and incubated overnight in buffer containing 5% skim milk powder at 4 °C. The two corresponding antibodies were then added and the incubation was continued for 1 h at room temperature. Electrochemiluminescence imaging was performed, using β-actin as an internal reference.

Histological observation
Rats from each group were dissected at different time points. Hepatic hilar tissue was fixed in 10% formalin, embedded in paraffin, sectioned, and subjected to histological examination after staining with hematoxylin and eosin.

Statistical analyses
All statistical analyses were performed by GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA). One way analysis of variance was used for comparison of quantitative data between different groups. Least significant difference was used for further multiple comparison. Data are expressed as mean ± standard deviation (x ± s). A P < 0.05 was considered to be statistically significant.

RESULTS
Pathological examination
Pathological changes in liver tissues were examined in control and cirrhotic rats at 8 weeks after the establishment of the model. Liver tissue in control rats (Figure 1A) showed a well-defined architecture. The structure of the hepatic lobules was clear, the liver cells were arranged in cords around the central vein, the sinuses showed a regular structure, the nuclei of the liver cells were round, and there was no periportal inflammatory cell infiltration. However, in the experimental group (Figure 1B), there were widespread false lobules, visible intrahepatic, false lobular re-segregation of fibers, obvious nodules of stem cell regeneration, and prominent inflammatory cell infiltration around the portal area and central veins.

Human UCMSC culture
After culture and passing for three generations, human UCMSCs appeared fusiform and relatively homogeneous, and were arranged in parallel or whirlpool-like adherent growths (Figure 2). Flow cytometry after the third passage showed that the cells expressed high levels of surface markers characteristic of UC-MSCs, including CD73, CD90, and CD105 (97.2%, 99.7%, and 98.1%, respectively) (Figure 3). The cells were negative for CD34 (0.2%), CDHLA-DR (0.3%), CD79a (2%), CD14 (0.3%), and CD45 (0.5%).

Hepatic colonization by DiI-labeled human UCMSCs
After 24 h, DiI-labeled UCMSCs were seen in the livers of rats in the UCMSC and UCMSC plus Tan II A groups (average UCMSC counts, 25.4 ± 16.6 and 34.8 ± 22.0, respectively), but not in the control group (Figure 4). No fluorescent protein-labeled cells

<table>
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<th>Primer</th>
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<th>Product length (bp)</th>
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<tr>
<td>ALB-F</td>
<td>ACACCCAGAAAGGCACCTCAG</td>
<td>184</td>
</tr>
<tr>
<td>ALB-R</td>
<td>AAAGGCAATCAACACCAAGGC</td>
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</tr>
<tr>
<td>CK-18-F</td>
<td>CCGCTACGCCCTACAGAT</td>
<td>103</td>
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<td>CK-18-R</td>
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<td>103</td>
</tr>
<tr>
<td>Actin-F</td>
<td>CATTCCAAATATGAGATGGTTGT</td>
<td>103</td>
</tr>
<tr>
<td>Actin-R</td>
<td>TGTTGGACCTTGAGGAGGACT</td>
<td>103</td>
</tr>
</tbody>
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were observed in any group at 1 week after transplantation.

**Changes in serum ALB and ALT levels after UCSMC transplantation**

There were no obvious changes in serum ALB and ALT levels in any of the groups at 3 days after transplantation. However, at 1 and 3 months after transplantation, ALT levels were obviously decreased and ALB levels significantly increased in the UCSMC and UCSMC plus Tan II A groups, compared with levels in the control group ($P < 0.05$) (Figure 5).

**Expression of ALB and CK-18 mRNA and protein**

mRNA and protein expression levels of ALB and CK-18 were significantly higher in the UCSMC and UCSMC plus Tan II A groups compared with the control group, according to RT-PCR and western blot, respectively ($P < 0.05$) (Figures 6, 7).

**DISCUSSION**

In this study, we investigated the therapeutic effects of UCMSC transplantation and Tan IIA injection in a rat model of liver cirrhosis. Liver-injury repair is a two-stage process involving the regeneration of liver cells and hyperplasia of fibrous tissue. Fibrous-tissue hyperplasia is useful during the early stage of liver damage, but continued fibrosis results in the excessive accumulation of extracellular matrix, and eventually to cirrhosis and liver failure. 25-29 The treatment of liver cirrhosis thus requires an increase in the number of new liver cells and a decrease in the deposition of extracellular matrix. In this study, we used a histologically proven carbon tetrachloride-induced rat model of cirrhosis. Furthermore, human UCMSCs had colonized the rat livers 24 h after transplantation via the tail vein, as confirmed by immunofluorescence microscopy. Serum ALT levels were significantly lower and serum ALB levels significantly higher in the transplanted rats at 1 and 3 months after transplantation, compared with the control rats. These results are consistent with Zhang et al. 27 who showed that ALB and CK-18 mRNA and protein were expressed in the livers of rats transplanted with UCMSCs, and also with Banas et al. 30 who showed that UCMSCs could differentiate into liver cells in rats with liver cirrhosis. Stem-cell homing to a new organ and their differentiation into mature cells of the corresponding tissue can replace and repair injured tissues. However, increasing the number of transplanted cells for differentiation remains an important issue in transplantation treatment. This increase in cell number is currently achieved mainly by the application of chemical inducers of differentiation in vitro; however, most chemical inducers have some degree of toxicity. Recent experiments 31-33 have confirmed the potential of Tan IIA, a monomer isolated from S. miltiorrhiza, as an inducer of stem cell pro-

![Figure 4 Colonization of rat liver by Dil-labeled human UCMSCs (Dil x100)](image)
liferation and differentiation. Tan IIA has been used in vitro to induce bone marrow MSCs to differentiate into bone cells and neuron-like cells. We demonstrated that Tan IIA increased the number of transplanted UCMSCs colonizing the liver after 24 h, compared with UCMSC transplantation alone. Moreover, serum ALT levels were significantly lower and serum ALB levels significantly higher at 1 and 3 months after transplantation, and hepatic ALB and CK-18 mRNA and protein expression levels were higher in transplanted rats treated with Tan IIA, compared with UCMSCs alone. These results indicate that Tan IIA can influence the outcomes of human UCMSC transplantation in rats with liver cirrhosis, but the underlying mechanism remains unclear. Further studies are needed to determine if Tan IIA can induce the differentiation of UCMSCs into hepatocytes.

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