Original Article

Effect of bone marrow mononuclear cell homograft transplantation on ischemic type biliary lesion in rabbit livers

Zhaowei Qu1, Chengming Sun1, Bing Li1, Rujia Zhang2, Wei Dong1, Jianmin Sun1, Yubao Zhang1

1Department of Hepatobiliary and Pancreatic Surgery, The Affiliated Tumor Hospital of Harbin Medical University, Harbin, Heilongjiang Province, China; 2Operating Room, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, China

Received April 26, 2016; Accepted August 5, 2016; Epub September 15, 2016; Published September 30, 2016

Abstract: Ischemic type biliary lesion (ITBL) is frequently occurred after orthotopic liver transplantation and severely affects patients’ survival rate. The pathogenesis of ITBL is still unclear yet, but has been suggested to be related with multiple factors including ischemia time of donor liver, reperfusion damage and immune factors. Complete ischemia model of intrahepatic biliary tract was established in rabbit by combined blockage of common biliary tract and common hepatic artery. In transplantation group, mononuclear cells separated from bone marrow as infused via hepatic artery. Liver functions in two groups were compared. Both mRNA and protein expressions were determined by real-time PCR and Western blotting, respectively. After autograft transplantation of bone marrow derived mononuclear cells, liver function indexes including AST, ALT, ALP, total bilirubin and direct bilirubin were all decreased compared to control group (P<0.05). Both mRNA and protein expression levels of Bcl-2 and VEGF were increased while expression of Fas was decreased (P<0.05). Autograft transplantation of bone marrow derived mononuclear cells could prevent the occurrence of ITBL, via facilitating angiogenesis, improving apoptosis/anti-apoptosis balance and improving hepatic and biliary functions.

Keywords: Bone-marrow mononuclear cells, autograft transplantation, ischemic type biliary lesion

Introduction

After orthotopic liver transplantation, it is common to observe the occurrence of biliary complications, in which ischemic type biliary lesion (ITBL) is one of frequent diseases and severely affects survival rate and life quality of patients [1, 2]. In clinics it is extremely difficult to treat multifocal or diffused ITBL, as endoscopy, intervention or routine surgical procedure have barely any efficacy, leaving re-transplantation as the only option and thus increasing the percentage of secondary round of liver transplantation [3, 4]. With advancement in microcirculation of biliary tract, studies have shown that the blood supply of intrahepatic biliary tract comes from arterial network originating from branches of hepatic artery and peripheral vascular plexus of biliary tract [5]. The pathogenesis of intrahepatic ITBL is still unclear yet, probably due to multiple factors including extended time of heat/cold ischemia of donor liver, thrombosis of peripheral vascular plexus after reperfusion, secondary heat ischemia time of intrahepatic biliary tract, and immune injury [6, 7]. Among those factors, ischemia injury of graft is mostly correlated with post-operative ITBL. The destruction of hepatic artery branches and dysfunction of biliary duct peripheral arterial plexus may all lead to ischemia, necrosis and cirrhosis of biliary tract, leading to intrahepatic ITBL [8, 9].

Bone marrow mononuclear cells (BM-MNCs) are major components of bone marrow, including hematopoietic stem cells, mesenchymal stem cells and endothelial progenitor cells [10], all of which play a synergistic role in facilitating angiogenesis and vascular repair. When being used as the donor cell for transplantation, BM-MNCs could participate in the formation of adult vessels, thus becoming a research focus...
Mononuclear cell and ITBL

in ischemia disease as the angiogenesis treatment [11, 12]. Multiple researches have suggested that BM-MNCs can be differentiated into new vessels and can repair vascular injury, in addition to the secretion of various angiogenesis-facilitating factors including vascular endothelial growth factor (VEGF) [13, 14]. The functional role and mechanism of BM-MNCs in preventing ITBL, however, has not been illustrated.

Materials and methods

Animals

A total of 20 Japanese white rabbits (10 males and 10 females, 3 month old, body weight 2–2.5 kg) were purchased from laboratory animal center of Harbin Medical University. Animals were singly housed in a temperature/humidity-regulated facility (21±1°C, 50%–70%) with 12 h dark/light cycle. After 1-week acclimation, animals were randomly divided into control and transplantation group (N=10). ITBL model was then established in both groups, of which transplantation group received BM-MNCs treatment afterwards.

Rabbits were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the Affiliated Tumor Hospital of Harbin Medical University.

Reagents and instruments

Surgical instruments were purchased from Suzhou Medical Instrument Factory. Surgical microscope was purchased from Zhenjiang Optical Instrument Company. DMEM/F12 culture medium fetal bovine serum (FBS), EDTA and penicillin/streptomycin dual antibiotics were produced by Hyclone, US. Lymph cell separation buffer was purchased from Haoyang Biological Company. HCl-procaine injection fluid was produced by Tianjin Pharmaceutical Company. DMSO and MTT powders were purchased from Gibco, US. Trypsin-EDTA digestion buffer was purchased from Sigma, US. PVDF membrane was purchased from Pall Life Sciences, US. Western blotting reagents were purchased from Beyotime Bioengineering Company. ECL reagent was purchased from Amersham Biosciences. Mouse anti-rabbit Bcl-2, Fas and VEGF monoclonal antibody, and goat anti-mouse IgG labelled with horseradish peroxidase (HRP) were produced by Cell Signaling, US. DNA amplified was produced by PE, US (model, Gene Amp PCR System 2400). Other common reagents were purchased from Sangon, Shanghai. RNA extraction kit and reverse transcription kit were purchased from Axygen, US.

ITBL model in rabbit liver

Rabbits were anesthetized by 40 mg HCl-procaine. Liver tissues were exposed, followed by the ligation of hepatic common artery and biliary common duct, along with peripheral vascular plexus around biliary duct. Mesenchymal tissues between biliary common duct/hepatic common artery and portal vein were also removed to block any potential blood supply for biliary duct. 2 h later, artery clips were removed to recover blood supply, in generating intrahepatic ITBL model [15].

BM-MNCs separation and transplantation

Approximate 8 ml bone marrow was drawn from rabbit iliac bones under sterilized bone puncture needle. After saline rinsing, cell suspensions were mixed with 10 ml DMEM/F12 complete culture medium for 2500 rpm centrifugation for 20 min. Supernatants were removed and precipitations were separated for BM-MNCs using lymph cell separation buffer. Those ITBL rabbits were then anesthetized for exposing abdominal cavity, following injection of 2 ml autograft BM-MNCs (2 × 10⁷ per ml) via hepatic common artery puncture.

Sample collection

2 weeks after surgery, blood samples were collected from abdominal aorta. After incubating under room temperature for 30 min, blood samples were centrifuged at 3600 rpm for 10 min to obtain supernatants, which were frozen for further use. All rabbits were then sacrificed to obtain biliary duct tissues for further use.

Liver function indexes

Liver function indexes including AST, ALT and ALP, total bilirubin and direct bilirubin were quantified by automatic biochemical analyzer.

Real-time PCR

Biliary duct tissues were homogenized and mixed with lysis buffer on ice for 15 min incubation. Trizol reagent was used to extract total
mRNA. Reverse transcription was performed following manual instruction. Primer 6.0 was used to design primers of all target genes and was synthesized by Yingjun Biotech Cooperation (see Table 1 for sequences). Real-time PCR assay was performed under following conditions: (1) 55°C for 1 min, followed by 35 cycles each containing 92°C 30 s, 58°C 45 s and 72°C 35 s (for GAPDH, Fas and VEGF genes); and (2) 55°C for 1 min, followed by 35 cycles each containing 92°C 30 s, 57°C 30 s and 72°C 35 s (for Bcl-2 gene). CT values of samples and standards were calculated based on collected fluorescent intensity values collected by PCR amplifier. A standard curve was then plotted for semi-quantitative analysis using 2^−ΔCt method.

**Western blotting**

Total tissue proteins were extracted by tissue homogenization and lysis buffer incubation on ice for 15~30 min. Ultrasonic rupture was then performed (5 s × 4 times) followed by 10,000 g centrifugation for 15 min. Supernatants were saved for quantification of proteins, which were separated in 10% SDS-PAGE. Proteins were then transferred to PVDF membrane by semi-dry method. Non-specific binding sites were removed by 5% defatted milk powder for 2 h. Mouse anti-rabbit Bcl-2, VEGF or Fas monoclonal antibody (1:1,000) was added for 4°C overnight incubation. On the next day, the membrane was washed in PBST, and incubated with 1:2,000 goat anti-mouse secondary antibody for 30 min incubation. ECL substrate was then added for 1 min development followed by X-ray exposure. Protein bands were processed by Quantity One software for measuring optical intensity of protein bands (N=4 for each group) for further analysis.

**Statistical analysis**

SPSS19.0 software was used to process all collected data. Measurement data were expressed as mean ± standard deviation (SD). Between-group-comparison was performed by student t-test. A statistical significance was defined when P<0.05.

**Results**

**General conditions of animals**

All rabbits in both groups survived until the endpoint. In ITBL control (model) group, rabbits had decreased activity, mental retard, dispersed furs, bad appetite, decreased food and water intake, and slow increase or even decrease in body weight. Transplantation group, however, had better mental recovery, increased motor activity, water/food intake, shiny fur and higher body weight (P<0.05 compared to control group, Table 2).

**Liver function indexes**

2 weeks after surgery, liver function indexes including AST, ALT, ALP, total bilirubin and direct bilirubin were all measured. As shown in Table 3, after BM-MNCs transplantation, liver enzymes AST, ALT, biliary enzyme index ALP were all decreased, along with lower total bilirubin and direct bilirubin levels, as compared to control group (P<0.05).

**mRNA level of rabbit Bcl-2**

Real-time PCR was used to detect the effect of BM-MNCs transplantation on mRNA expression of Bcl-2 in rabbits. Results showed that after transplantation, the expression of anti-apoptotic factor Bcl-2 was significantly elevated compared to control group (P<0.05, Figure 1).

**Fas mRNA expression**

We further checked the effect of autograft transplantation of BM-MNCs on mRNA level of Fas in ITBL model rabbits. As contrast to those for Bcl-2, apoptotic factor Fas was significantly
Mononuclear cell and ITBL

Table 3. Liver function indexes

<table>
<thead>
<tr>
<th></th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
<th>Total bilirubin (µmol/L)</th>
<th>Direct bilirubin (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>278.5±6.9</td>
<td>99.1±10.1</td>
<td>179.3±12.9</td>
<td>20.8±3.8</td>
<td>13.9±2.1</td>
</tr>
<tr>
<td>Transplantation</td>
<td>161.3±7.9*</td>
<td>42.1±9.8*</td>
<td>110.2±17.3*</td>
<td>9.3±1.5*</td>
<td>4.5±1.2*</td>
</tr>
</tbody>
</table>

Note: *, P<0.05 compared to control group.

Figure 1. Effect of BM-MNCs transplantation on Bcl-2 mRNA. *, P<0.05 compared to control group.

Figure 2. Effect of BM-MNCs transplantation on Fas mRNA. *, P<0.05 compared to control group.

Figure 3. Effect of BM-MNCs transplantation on VEGF mRNA. *, P<0.05 compared to control group.

Figure 4. Protein expressions of Bcl-2, Fas and VEGF. A: Control group; B: BM-MNCs transplantation group.

VEGF mRNA level

We further checked the effect of autograft transplantation of BM-MNCs on mRNA level of VEGF in ITBL model rabbits by RT-PCR. Results showed that, after transplantation, VEGF mRNA level was significantly up-regulated (P<0.05 compared to control group, Figure 3).

Protein expression of Bcl-2, Fas and VEGF

To further illustrate the expression of Bcl-2, Fas and VEGF in ITBL, and the effect of BM-MNCs transplantation, we used Western blotting to detect the levels of those proteins. Results showed that similar to those in mRNA levels, transplanted rabbits had increased protein levels of Bcl-2 and VEGF, and decreased Fas (P<0.05 compared to control group, Figures 4 and 5).

Discussion

Intrahepatic ITBL can lead to the narrowing of biliary duct, intrahepatic cholestasis, and further biliary duct injury or even liver failure. ITBL is especially common in liver transplantation patients, and can induce liver dysfunction and...
Mononuclear cell and ITBL

![Figure 5. Effects of BM-MNCs transplantation on protein levels of Bcl-2, Fas and VEGF. * P<0.05 compared to control group.](image)

rejection after surgery, and sometimes requires a secondary liver transplantation due to complete loss of liver function [1, 16]. However, exact pathogenesis mechanism of ITBL is still unclear. It is commonly believed that the long-term ischemia and reperfusion injury of extrahepatic biliary duct could lead to ischemic injury of biliary duct, leading to the formation of microthrombosis of hepatic artery, thus impeding the blood supply for biliary duct and injury of peripheral vascular plexus, all of which can cause ischemia and death of biliary duct cells, leading to ITB [17]. Currently no effective method can reverse the progression of ITBL, neither does specific treatment of disease. As it cause higher failure rate of transplantation, it is thus necessary to effectively treat intrahepatic biliary duct lesion.

BM-MNCs contain bone marrow stem cells for secreting multiple angiogenesis facilitating factors such as VEGF, thus playing a crucial role in treating ischemia disease [10-14]. Some study has proved that after the occurrence of ischemic heart stroke, transplantation of autograft MNCs could significantly improve blood ejection fraction, ventricular wall motility and reperfusion of ischemic tissues [19]. In another model of limb ischemia by ligation of left common iliac artery, transplantation of autograft BM-MNCs into leg muscles can induce the further differentiation into endothelial, vascular smooth muscle, skeletal muscle and adipocytes tissues. At the site of transplantation, microvessel density (MVD), density of small artery and blood flow rate of femoral were all increased. After stroke, the transplantation of BM-MNCs also can help to form new vessels around ischemic boundary zone. In both clinical and basic study, the purification of BM-MNCs has well defined protocols with timely manner, thus can be used during liver transplantation [20, 21]. The functional role and mechanism of xenograft BM-MNC transplantation in ITBL are still unknown yet. Our study found that autograft transplantation of BM-MNCs improved general condition of animals, which also had improved liver function indexes including AST, ALT, ALP and bilirubin, indicating that it could facilitate the functional recovery of biliary duct. Further results showed that BM-MNCs could facilitate the expression of anti-apoptotic factors and inhibit apoptotic factor expression, to alleviate the injury caused by intrahepatic MNCs. Meanwhile, such transplantation could facilitate the proliferation/differentiation of vascular endothelial cells, speed the formation of novel vessels, for ultimate goal of improving vascular damage after intrahepatic ITBL.

In summary, the transplantation of autograft BM-MNCs could prevent ITBL, possibly via facilitating angiogenesis, modulating apoptosis/anti-apoptosis homeostasis. It can also improve liver/biliary function. As one counter-measure with easy and efficacy, autograft transplantation of bone marrow could be used in clinics to prevent the occurrence of intrahepatic ITBL in clinics.

Acknowledgements

Scientific Research Fund of Heilongjiang Provincial Education Department (12531357).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yubao Zhang, Department of Hepatobiliary and Pancreatic Surgery, The Affiliated Tumor Hospital of Harbin Medical University, No. 150, Haping Road, Nangang District, Harbin 150081, Heilongjiang Province, China. Tel: +86-0451-86298082; Fax: +86-0451-86298082; E-mail: zhangyubaao@163.com

References

Mononuclear cell and ITBL


