Bone Marrow-Derived Mesenchymal Stem Cells Promote Hepatic Regeneration after Partial Hepatectomy in Rats

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Key Words

Mesenchymal stem cell · Partial hepatectomy · Liver regeneration

Abstract

Objectives: Our goal was to study the ability of mesenchymal stem cells (MSCs) to stimulate liver regeneration after partial hepatectomy in rats. **Methods:** MSCs were isolated from bone marrow and cultured in vitro. Their characteristics were analyzed by flow cytometry. After 70% partial hepatectomy, Sprague-Dawley rats were randomly divided into three groups: a control group that was injected with saline, animals that received bone marrow-derived MSCs (BM-MSCs) by tail vein injection (the BM-MSC-TV group) and animals that received BM-MSCs by portal vein injection (the BM-MSC-PV group). The injected BM-MSCs were traced by labeling with 4',6-diamidino-2-phenylindole, and cell proliferations were determined by immunohistochemical staining with Ki-67 and 5-bromo-2'-deoxyuridine. **Results:** After the third passage, the cultured BM-MSCs had a fibroblast-like morphology and expressed high levels of stem cell markers CD29 and CD90. The levels of albumin rose significantly in the BM-MSC-TV and BM-MSC-PV groups compared with the control group. The number of 4',6-diamidino-2-phenyl-

indole-positive liver cells in the BM-MSC-PV group was significantly higher than in the BM-MSC-TV group. The levels of Ki-67 and 5-bromo-2'-deoxyuridine were significantly higher in the BM-MSC-TV and the BM-MSC-PV groups than in the controls. **Conclusion:** Taken together, these results indicate that BM-MSC injections enhance liver regeneration after partial hepatectomy in rats.

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Introduction

As a vital organ, the liver has the remarkable feature of regeneration. Hepatitis virus infection, inherited metabolic disease, drug abuse and trauma cause liver injury and can induce hepatic failure. In these situations, liver regeneration is triggered rapidly to restore hepatic function, and hepatic stem cells play an important role in regeneration [1]. Hepatic stem cells in liver tissues have high proliferation potential in vitro and can be induced to differentiate into hepatocytes [2, 3]. However, hepatocyte proliferation may be inhibited if the injury is too

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severe [4], and liver regeneration is often compromised after surgery for malignancies [5, 6]. Poor liver regeneration might result from insufficient hepatic stem cells and/or their late activation. To overcome these limitations, the injection of stem cells in sufficient numbers might be a feasible strategy to restore normal liver functions.

Bone marrow stem cells, including hematopoietic stem cells and bone marrow-derived mesenchymal stem cells (BM-MSCs), are pluripotent and can self-renew. Recently, BM-MSCs have been differentiated into neurons [7], cardiomyocytes [8], endothelial cells [9], chondrocytes [10] and hepatocytes [11]. BM-MSCs can differentiate into hepatocytes in vitro [12] and in vivo [13] and also engraft on injured tissue and recover the function of injured tissue [14, 15]. Furthermore, autologous BM-MSC therapy has shown great promise in enhancing tissue regeneration in a range of acute and chronic disease [16], including liver disease [17, 18]. Although Kaibori and his colleagues [19] reported that BM-MSCs could stimulate liver regeneration after hepatectomy in mice, BM-MSCs were not characterized in their study. In this study, we clarified the role of BM-MSCs in liver regeneration after partial hepatectomy.

Materials and Methods

Animals

Male Sprague-Dawley rats were purchased from SLAC Laboratory Animal Co., Ltd., Shanghai, China, and maintained in isolated ventilated cages under well-controlled conditions at 23°C, 55–60% humidity, with ventilation >1.27 m³/h and a 12-hour light-dark cycle. Animal experiments were performed in compliance with federal Chinese laws and the animal guidelines of Fujian Medical University.

Isolation and Culture of BM-MSCs

BM-MSCs were harvested from the femurs and tibias of Sprague-Dawley rats (60–80 g) and suspended in complete DMEM/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (PAA Laboratories, Austria), 100 U/ml penicillin and 100 mg/l streptomycin. The BM-MSCs were then filtered through a 200-μm mesh and suspended in complete DMEM/F12 medium. These cells were cultured at 37°C with 5% CO₂, and medium was replaced every 2–3 days. By 7–10 days, when the cell confluence reaches 80–90%, cells were passaged. Subsequently, the cells were split at a 1:2 dilution every 3 days. Cells from the third passage were used in experiments presented.

Flow Cytometry Analysis

Cells from the third passage were trypsinized and washed twice with PBS. Cells (10⁶/100 μl) were incubated with antibodies against CD29, CD45, CD90 (Biolegend, USA) and CD34 (Santa Cruz, Calif., USA) for 40 min at room temperature. After incubation,

cells were washed with PBS twice and resuspended in 2 ml of PBS for analysis. Coulter flow cytometry was used to analyze the surface markers of the BM-MSCs.

Labeling with 4',6-Diamidino-2-Phenylindole

When the confluence of BM-MSCs (third passage) reached 60–70%, 4',6-diamidino-2-phenylindole (DAPI; 1 μg/ml) was added to the medium and cells were cultured for 12 h. After incubation, cells were washed with PBS 6 times and a cell sample was examined by fluorescence microscopy. For identifying DAPI+ BM-MSCs in liver, the liver was harvested on day 9 after surgery and frozen sections were prepared. The DAPI-labeled cells were counted under a fluorescence microscope.

Partial Hepatectomy and Administration of BM-MSCs

Male Sprague-Dawley rats (180–220 g) were anesthetized with ether. After midline laparotomy, the pedicle of the left lateral and median lobes of the liver were ligated and resected (70% partial hepatectomy). Suturing of the peritoneum and skin were performed independently. Twenty-four hours after hepatectomy, these rats were divided into three groups: in one group, BM-MSCs (1.5 × 10⁶) were injected into the portal vein (BM-MSC-PV group); in the second group, BM-MSCs (1.5 × 10⁶) were injected into the tail vein (BM-MSC-TV group); in the control group, saline was injected into the tail vein.

Biochemical Analysis of Blood Samples

On days 3 and 9 after surgery, the blood samples were harvested from the retro-orbital plexus (1–2 ml) and the inferior vena cava (1–5 ml), respectively. The plasma was isolated and alanine transaminase (ALT) and albumin were measured by standard laboratory methods.

Immunohistochemical Analysis of Liver Tissue

Two hours before sacrifice (9 days after surgery), rats were intraperitoneally injected with 50 mg/kg 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, USA). After being anesthetized by intraperitoneal injection with 10% chloral hydrate (0.3 ml/100 g), liver tissues were removed and fixed with 10% formalin for 24 h. Tissue sections 4- to 7-μm thick were prepared. After antigen retrieval, endogenous peroxidase was blocked by incubation with 3% H₂O₂ for 10 min. After washing with PBS, slides were blocked with normal goat serum for 30 min. Following the addition of mouse anti-BrdU antibody (Zymed Laboratories, USA) diluted 1:600, sections were incubated for 1 h. After washing, slides were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody for 30 min. Then slides were incubated with DAB solution for 3–10 min and counterstained with hematoxylin. For Ki-67 staining, the staining procedure was the same as the BrdU staining, except that the rabbit anti-rat Ki-67 antibody (Abcam, USA) was used at a 1:50 dilution as primary antibody, and horseradish peroxidase-conjugated goat anti-rabbit antibodies (Gold Bridge, China) were used as the secondary antibody. A clear brown color around the cell nucleus was considered as positive. Ten high-power fields (×400) per sample were analyzed.

Statistical Analysis

Continuous variables were compared by one-way analysis of variance. When a significant difference between groups was apparent, multiple comparisons of means were performed using Stu-

Fig. 1. Morphology of BM-MSCs. BM-MSCs isolated from rats were cultured in vitro and the cell morphology was observed using an inverted microscope. **a** BM-MSCs 4 days after plating. **b** BM-MSCs showed a fibroblastic phenotype 3 days after the third passage. $\times 100$.

Fig. 2. Characteristics of BM-MSCs. BM-MSCs from the third passage were stained with the indicated antibody and analyzed by flow cytometry. **a** BM-MSCs are shown as a dot plot. The expression levels of CD34 (**c**), CD45 (**d**), CD29 (**e**) and CD90 (**f**) of BM-MSCs are presented as a histogram. Relative to the isotype antibody (**b**), the percentage of expression of the indicated markers was defined as the B region. FS = Forward scatter; SS = side scatter; PE = phycoerythrin.

dent-Newman-Keul's test. Data are presented as means $\pm$ standard deviations. Categorical data analyses were performed using Fisher's exact test. All statistical assessments were two-sided and evaluated at the 0.05 level of difference in significance. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS Inc., Chicago, Ill., USA).

Results

Isolation and Characteristic of Rat BM-MSCs

Morphologically freshly isolated BM-MSCs were oval in shape (fig. 1a). They attached to culture dishes and grew slowly in the first few days. At the third passage,

the BM-MSCs presented a fibroblast-like morphology (fig. 1b). We further assessed the phenotypic characteristics of these cells by flow cytometry. As shown in figure 2, 98.6% of the cells expressed the integrin- β antigen CD29 and 99.7% of the cells expressed the stem cell marker CD90, but very few cells expressed the adhesion/stem cell marker CD34 (0.3%) or CD45 (0.6%). These phenotypes of culture-expanded BM-MSCs conformed to the criteria for MSCs [20, 21].

Injection of BM-MSCs after Partial Hepatectomy

To determine the regenerative capabilities of BM-MSCs, we injected these cells into a partial hepatectomy

BM-MSCTV

BM-MSCPV

Fig. 3. Detection of DAPI-labeled BM-MSCTV in damaged liver. Nine days after hepatectomy, rats were sacrificed and livers were frozen and sectioned. The DAPI-labeled BM-MSCTV were observed by fluorescence microscope. $\times 100$.

Table 1. Serum levels of ALT and albumin in rats subjected to partial hepatectomy at days 3 and 9 after injection of BM-MSCTV

Groups	n	Day 3		Day 9	
		ALT, U/l	ALB, g/l	ALT, U/l	ALB, g/l
Saline	10	95.2 $\pm$ 28.3	30.3 $\pm$ 3.6	73.6 $\pm$ 19.1	29.2 $\pm$ 4.9
BM-MSCTV	9	105.5 $\pm$ 40.8	24.2 $\pm$ 11.9	53.2 $\pm$ 21.9	35.1 $\pm$ 3.5*
BM-MSCPV	9	150.7 $\pm$ 86.8	27.4 $\pm$ 2.0	73.5 $\pm$ 19.0	34.1 $\pm$ 2.6*

Results are shown as the mean $\pm$ standard deviation. ALB = Albumin. * $p < 0.05$, significant difference versus the saline group.

rat model. In the BM-MSCPV animals, 1 rat bled to death and 9 rats survived after the injections. In the BM-MSCTV group, 9 rats survived after injection and only 1 rat had breathing problems that resulted in death. All rats survived saline injection (10/10). Five days after hepatectomy, 3 rats in the saline group (3/10) and 1 rat in the BM-MSCPV group (1/9) had enlarged abdomens. Nine days after the hepatectomies, rats were sacrificed and ascites were examined in those rats with enlarged abdomens. The results showed that 3 rats in the saline group and 1 rat in the BM-MSCPV group had ascites. No ascites were observed in the BM-MSCTV group (0/9). There was no significant difference in the incidences of ascites 5 days after hepatectomy among the 3 groups ($p = 0.288$).

Liver Function after BM-MSCTV Injection

Three days after hepatectomy, the levels of ALT were slightly higher in the BM-MSCPV (150.7 $\pm$ 86.8 U/l) and BM-MSCTV (105.5 $\pm$ 40.8 U/l) groups than in the saline (95.2 $\pm$ 28.3 U/l) group, whereas the levels of albumin were lower in the BM-MSCPV (27.4 $\pm$ 2.0 g/l) and BM-MSCTV (24.2 $\pm$ 11.9 g/l) groups than in the saline con-

trol group (30.3 $\pm$ 3.6 g/l), but the difference was not statistically significant. Nine days after hepatectomy, the levels of ALT in the BM-MSCTV (53.2 $\pm$ 21.9 U/l) and BM-MSCPV (73.3 $\pm$ 19.0 U/l) groups decreased towards the levels seen in the saline control group (73.6 $\pm$ 19.1 U/l). However, the levels of albumin were significantly elevated ($p < 0.05$) in the BM-MSCTV-injected groups (BM-MSCTV 35.1 $\pm$ 3.5 g/l, and BM-MSCPV 34.1 $\pm$ 2.6 g/l) when compared with the saline control group (29.2 $\pm$ 4.9 g/l). There was no statistically significant difference between the BM-MSCTV group and the BM-MSCPV group (table 1).

Tracing of Injected BM-MSCTV

To trace the injected cells, BM-MSCTV were labeled with DAPI before injection (fig. 3). Nine days after injection, high levels of DAPI-labeled BM-MSCTV were observed in the BM-MSCPV group (18.1 $\pm$ 3.4) when compared with the BM-MSCTV group (7.6 $\pm$ 2; $p < 0.001$).

Expression of Ki-67 and DNA Synthesis in Damaged Liver

To examine the proliferative activity of the injected BM-MSCTV, immunohistochemical staining for the proliferation indicator, Ki-67, was performed. As shown in figure 4, 9 days after hepatectomy, many Ki-67+ nuclei were observed in the BM-MSCTV-injected groups (BM-MSCTV 103.3 $\pm$ 40.4 cells, and BM-MSCPV 95.8 $\pm$ 26.6 cells), more than in the saline group (60.2 $\pm$ 35.7 cells; $p = 0.023$). To further confirm the proliferation of hepatocytes, we analyzed DNA synthesis by BrdU staining after BM-MSCTV injection (fig. 5). Nine days after hepatectomy, the levels of BrdU+ cells were higher in the BM-MSCTV-injected groups (BM-MSCTV 15.9 $\pm$ 3.0 cells, and BM-MSCPV 16.0 $\pm$ 3.3 cells) than in the saline group (11.6 $\pm$ 3.7 cells; $p = 0.01$).

Fig. 4. Expression of Ki-67 in damaged liver. Nine days after hepatectomy, the livers were removed and the expression of Ki-67 was determined by immunohistochemical staining. $\times 400$.

Fig. 5. Effects of BM-MSCs on hepatocyte proliferation. Nine days after hepatectomy, the livers were processed for BrdU levels immunohistochemically to quantify hepatocyte proliferation. Representative micrographs of hepatocyte proliferation in liver tissue are shown. A small intestine sample showing high proliferation was used as a positive control.

Discussion

MSCs, the major stem cells in bone marrow, constitute the microenvironment of the bone marrow, regulate hematopoietic function and differentiate into various cells, including hepatocytes [10, 11, 22–24]. BM-MSCs are easily isolated from bone marrow, readily cultured in vitro

and can be used for autologous transplantation. Thus, they may be ideal seed cells for the treatment of injured tissue. The use of BM-MSCs is being explored in the fields of regenerative medicine and tissue engineering.

The hepatic microenvironment provides key factors that promote the differentiation of MSCs into hepatocyte-like cells that have the ability to recover normal liver function.

Arikura and his colleagues [25] immediately injected normal BM-MSCs through the portal vein after 70% partial hepatectomy of albumin-deficient rats. Four weeks after the injections, the expression of albumin mRNA and protein was seen in the liver of the albumin-deficient rats. Furthermore, albumin was present in serum. While Abdel Azizet et al. [14] isolated CD29+ MSCs from the bone marrow of males and injected them into the tail vein in a female rat fibrosis model, they found that the MSCs could differentiate into hepatocyte-like cells and reduce fibrosis by decreasing the precipitation of collagen. These results support the role of MSCs as a therapeutic agent for liver disease. In contrast, Cantz et al. [26] reported that differentiation of hepatocytes and regeneration were not observed after injection of MSCs. Therefore, the role of MSCs needs to be further clarified for liver disease therapy.

In this study, DAPI-labeled BM-MSCs injected through either the portal vein or tail vein were traced after 70% hepatectomy in rats. Higher amounts of DAPI+ BM-MSCs were observed in the BM-MSC-PV group than in the BM-MSC-TV group, suggesting that the injection route influences the homing of BM-MSCs. However, both portal vein and tail vein injection showed similar effects in enhancing liver regeneration and the recovery of normal liver function. There was no positive correlation between liver regeneration and the amount of homing BM-MSCs. Probably, 9 days was too short to analyze the capacity of homing BM-MSCs to differentiate into hepatocyte-like cells. The other possibility to explain this result is that MSCs could stimulate the proliferation of hepatocytes to promote liver regeneration in the injured liver.

The repopulation efficiency of injected cells after liver injury is another issue. Embryonic stem cell-derived hepatocytes have shown low repopulation efficiency in re-

cipient livers [27–29]. These results suggest that using well-differentiated hepatocytes for transplantation still causes some problems. The treatment of BM-MSCs after 70% hepatectomy might face a similar problem. However, administration of a growth factor or nitric oxide donor, such as insulin-like growth factor 1 [30] and sodium nitroprusside [31], to increase the differentiation and repair ability of BM-MSCs after transplantation might overcome this problem. Therefore, using progenitor cells which could migrate and differentiate into hepatocytes at the liver might be an alternative strategy for the treatment of liver injury.

Hepatocytes play a major role in liver regeneration after partial hepatectomy. Oval cells derived from the liver and bone marrow have been demonstrated to be hepatic stem cells [32–34], can be induced to differentiate into hepatocytes and are responsive to liver toxicity to promote liver regeneration. It has also been suggested that bone marrow cells could differentiate into hepatocytes after severe liver injury. When serious liver injury occurs, bone marrow stem cells quickly migrate to the liver and differentiate into hepatocytes [23]. In this study, the injected BM-MSCs could migrate to the damaged liver and might differentiate into hepatocytes to promote liver regeneration after partial hepatectomy.

In conclusion, we have demonstrated the effect of BM-MSCs after partial hepatectomy in rats. Autologous stem cells might provide a promising therapeutic effect on liver regeneration after surgery or liver injury.

Acknowledgement

This study was supported by the Key project of Fujian Province Science and Technology Plan (2011Y0043).

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