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Basic Study

Transplantation of bone marrow-derived endothelial progenitor cells and hepatocyte stem cells from liver fibrosis rats ameliorates liver fibrosis

Ling Lan, Ran Liu, Ling-Yun Qin, Peng Cheng, Bo-Wei Liu, Bing-Yong Zhang, Song-Ze Ding, Xiu-Ling Li

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Abstract**AIM**

To explore the effectiveness for treating liver fibrosis

by combined transplantation of bone marrow-derived endothelial progenitor cells (BM-EPCs) and bone marrow-derived hepatocyte stem cells (BDHSCs) from the liver fibrosis environment.

METHODS

The liver fibrosis rat models were induced with carbon tetrachloride injections for 6 wk. BM-EPCs from rats with liver fibrosis were obtained by different rates of adherence and culture induction. BDHSCs from rats with liver fibrosis were isolated by magnetic bead cell sorting. Tracing analysis was conducted by labeling EPCs with PKH26 *in vitro* to show EPC location in the liver. Finally, BM-EPCs and/or BDHSCs transplantation into rats with liver fibrosis were performed to evaluate the effectiveness of BM-EPCs and/or BDHSCs on liver fibrosis.

RESULTS

Normal functional BM-EPCs from liver fibrosis rats were successfully obtained. The co-expression level of CD133 and VEGFR2 was $63.9\% \pm 2.15\%$. Transplanted BM-EPCs were located primarily in/near hepatic sinusoids. The combined transplantation of BM-EPCs and BDHSCs promoted hepatic neovascularization, liver regeneration and liver function, and decreased collagen formation and liver fibrosis degree. The VEGF levels were increased in the BM-EPCs (707.10 ± 54.32) and BM-EPCs/BDHSCs group (615.42 ± 42.96), compared with those in the model group and BDHSCs group ($P < 0.05$). Combination of BM-EPCs/BDHSCs transplantation induced maximal up-regulation of PCNA protein and HGF mRNA levels. The levels of alanine aminotransferase (AST), aspartate aminotransferase, total bilirubin (TBIL), prothrombin time (PT) and activated partial thromboplastin time in the BM-EPCs/BDHSCs group were significantly improved, to be equivalent to normal levels ($P > 0.05$) compared with those in the BDHSC (AST, TBIL and PT, $P < 0.05$) and BM-EPCs (TBIL and PT, $P < 0.05$) groups. Transplantation of BM-EPCs/BDHSCs combination significantly reduced the degree of liver fibrosis (staging score of 1.75 ± 0.25 vs BDHSCs 2.88 ± 0.23 or BM-EPCs 2.75 ± 0.16 , $P < 0.05$).

CONCLUSION

The combined transplantation exhibited maximal therapeutic effect compared to that of transplantation of BM-EPCs or BDHSCs alone. Combined transplantation of autogenous BM-EPCs and BDHSCs may represent a promising strategy for the treatment of liver fibrosis, which would eventually prevent cirrhosis and liver cancer.

Key words: Bone marrow; Endothelial progenitor cells; Liver stem cell; Cell transplantation; Liver fibrosis

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Core tip: In addition to liver regeneration, hepatic neovascularization and endothelial/sinusoidal remodeling are critical factors for the treatment of liver fibrosis. Bone marrow-derived endothelial progenitor cells (BM-EPCs) have been shown to play a role in hepatic angiogenesis and to be beneficial in liver fibrosis. However, BM-EPCs are all derived from healthy individuals in recent studies. Here, we evaluated the feasibility of obtaining normal BM-EPCs from rats with liver fibrosis, and the effectiveness of combined transplantation of BM-EPCs and bone marrow-derived hepatocyte stem cells for treating liver fibrosis in order to achieve the dual effects of hepatic neovascularization and liver regeneration.

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INTRODUCTION

Liver fibrosis is the scarring process involved in the response of liver to injury. Most hepatocellular carcinoma, which is among the leading causes of cancer death worldwide, develops in the context of severe liver fibrosis and cirrhosis. Liver fibrosis is a necessary stage for cirrhosis and liver cancer. Bone marrow stem cells (BMSCs) have been recently used to treat liver fibrosis; whereas, BMSCs transplantation is not an effective treatment for liver fibrosis, due to the histopathological characteristics of liver fibrosis including deposition of excessive extracellular matrix, remodeling of abnormal vascular and sinusoid constriction^[1-6]. Low levels of engraftment and proliferation of exogenous cells in the liver parenchyma^[4,7], inhibition of endogenous liver regeneration^[4] and lack of a role in promoting hepatic neovascularization are the potential major drawbacks of BMSC transplantation therapy^[8].

Endothelial progenitor cells (EPCs) have been shown to play a function in angiogenesis and vasculogenesis^[9]. EPCs have been used for the treatment of liver fibrosis/cirrhosis in rats^[10-15]. Previous studies demonstrated that transplantation of bone marrow-derived EPCs (BM-EPCs) have a potential role in hepatic neovascularization, endothelial/sinusoidal remodeling and increased hepatic blood flow^[10,13]. However, BM-EPCs were all derived from healthy rats in these studies. In the present study, we sought to evaluate the feasibility of clinical application of autogenous EPCs therapy by using normal BM-EPCs obtained from liver fibrosis rats.

Furthermore, loss and destruction of functional

hepatocytes is another feature of liver fibrosis^[16]. BMSCs have been shown to restore and regenerate hepatic tissue^[17]. Our previous study demonstrated that β_2m^- /Thy-1⁺ bone marrow-derived hepatocyte stem cells (BDHSCs) derived from the pathoenvironment of liver injury in rats, which could be hepatocyte progenitors^[18], promoted hepatocyte proliferation and liver regeneration in liver fibrosis rats^[19]. Thus, a combined transplantation of BM-EPCs and BDHSCs may be more beneficial to liver fibrosis than transplantation of BM-EPCs or BDHSCs alone.

In the present study, we successfully obtained normal EPCs *in vitro* from bone marrow in liver fibrosis rats and evaluated the effectiveness of combined transplantation of BM-EPCs and BDHSCs *in vivo* for the treatment of liver fibrosis.

MATERIALS AND METHODS

Ethics and animals

Wistar rats (male, 8 wk, 250-300 g) (Animal Experiment Center of Henan Province, China) were housed in a standard animal laboratory. Animal studies were approved by the Animal Ethics Committee of Zhengzhou University and were in compliance with the Chinese National Regulations on the Use of Experimental Animals.

Isolation and culture of BM-EPCs and BDHSCs

The isolation and culture of BM-EPCs and BDHSCs were performed as described by Smadja *et al.*^[20] and Schatteman *et al.*^[21]. Wistar rats were subcutaneously injected with a 2:3 solution of carbon tetrachloride (CCl₄) and olive oil at a dose of 3 mL/kg body weight (double doses for the first time) twice per week for 6 wk to induce liver fibrosis. Bone marrow cells of rats with liver fibrosis were obtained by flushing femurs and humerus with DMEM/F12 medium (Gibco, New York, NY, United States). Bone marrow mononuclear cells (BMMCs) were then isolated by density gradient centrifugation with Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, United States) from bone marrow cells. After washing with red blood cell lysis buffer, BMMCs were seeded into culture flasks in DMEM/F12 medium supplemented with 10% fetal bovine serum (Gibco). After 24 h, the plastic-adherent cells were removed, and the nonadherent cells were collected, washed and replated into fibronectin-coated (10 μ g/mL; BD Biosciences, San Jose, CA, United States) culture flasks with the inducing medium containing DMEM/F12 medium supplemented with 10% fetal bovine serum, 20 ng/mL vascular endothelial growth factor (VEGF), 5 ng/mL basic fibroblast growth factor, 5 ng/mL epidermal growth factor (EGF) and 10 ng/mL insulin-like growth factor-1 (Peprotech, New Jersey, NJ, United States) and antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin). β_2m^- /Thy-1⁺ BDHSCs of rats with liver fibrosis were selected and purified by

magnetic bead cell sorting as previously reported^[18-22].

Flow cytometry for phenotypes of BM-EPCs

After 10 d of culture under inducing conditions, 2×10^6 BM-EPCs were incubated with the FcR blocking reagent (Miltenyi Biotec Inc., Auburn, CA, United States) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat CD133 antibody (BD Biosciences) and rabbit anti-rat vascular endothelial growth factor receptor 2 (VEGFR2) antibody (BD Biosciences) for 30 min at 4 °C, respectively. Then, cells were incubated with phycoerythrin (PE)-conjugated goat anti-rabbit secondary antibody (BD Biosciences) for 30 min at 4 °C. The phenotypic expression of BM-EPCs was analyzed by flow cytometry (FACS Scan flow cytometer; BD). BMMCs (2×10^6) cultured without induction for 10 d were used as a control.

Functional identification of BM-EPCs

BM-EPCs induced for 10 d were incubated with the inducing medium with 1,1'-dioctadecyl-3,3',3'-tetramethyl indocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (DiI-acLDL) (Molecular Probes, Eugene, OR, United States) for 4 h at 37 °C in the dark. The cells were fixed with 4% paraformaldehyde for 20 min and incubated with the inducing medium with FITC-conjugated ulex europaeus agglutinin I (FITC-UEA-I) (Sigma-Aldrich) for 1 h at 37 °C in the dark. The cells were photographed under a fluorescence microscope.

For the analysis of capillary tube formation, Matrigel (BD Biosciences, Heidelberg, Germany) was added to the wells of a pre-cooling 24-well plate at 0 °C, and allowed to solidify at 37 °C for 40 min. After 10 d of culture induction, BM-EPCs with the inducing medium were added into the wells (8×10^5 cells/well). Capillary tube formation on Matrigel was observed under an optical microscope after 24 h of incubation.

Cytokinetics of BM-EPCs

BM-EPCs induced for 10 d were seeded in a 96-well plate and divided into 3 groups: (1) the blank control group, containing only culture medium; (2) the experimental group, containing BM-EPCs from rats with liver fibrosis; and (3) the control group, containing BM-EPCs of normal rats. The cells were cultured in the incubator with 37 °C, 5% CO₂. 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-sulfophenyl)-2H-tetrazole monosodium salt (WST-8) (Neuron Science and Technology Development Co., Ltd., Beijing, China) was added to the plate (10 μ L/well, once/24 h) for 10 d. The values of optical density (OD) at 490 nm were detected by a microplate reader and the cytokinetics of BM-EPCs were analyzed.

Labeling and tracing of BM-EPCs in the liver

BM-EPCs were labeled with PKH26 (Sigma-Aldrich), according to the manufacturer's instructions. PKH26-

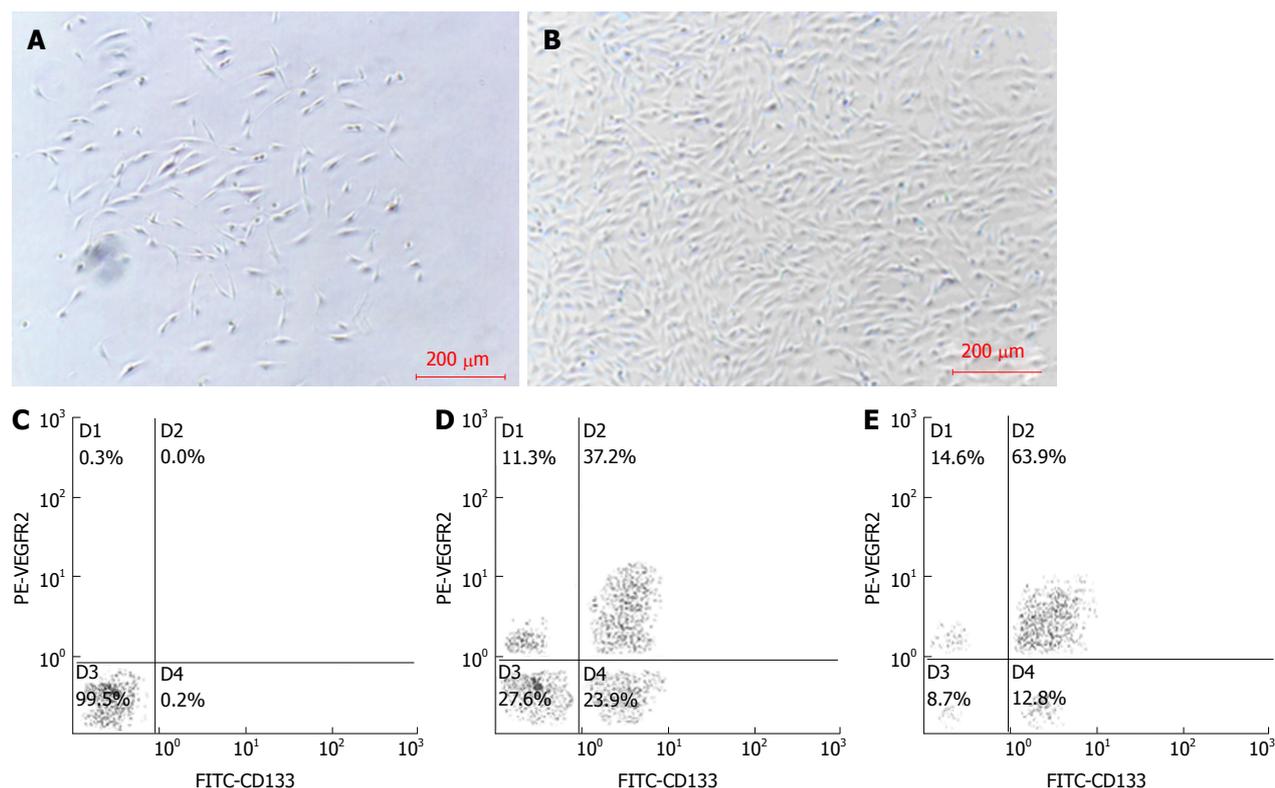


Figure 1 Morphology and phenotypes of BM-EPCs of rats with liver fibrosis, as shown by flow cytometry. A: Adherent cells cultured with induction for 4 d; B: Cell colonies cultured with induction for 10 d; C: Negative control without fluorescence-labeled BM-EPCs; D: Cells cultured without induction for 10 d; E: BM-EPCs cultured with induction for 10 d. Bars: 200 μm.

labeled BM-EPCs were transfused into rats *via* the tail vein after the rats were treated with CCl₄ for 3 and 5 wk. After 6 wk of modeling, the liver tissues were excised, and frozen sections were prepared. PKH26-positive cells were examined by using a fluorescence microscope. The liver sections of CCl₄-treated rats transfused with PBS served as a negative control.

CCl₄-induced liver fibrosis rat models and transplantation experiments

Liver fibrosis rat models were induced with CCl₄ as described above. The same volume of olive oil was used as a control. Forty rats were randomly divided into five groups (8 rats/group) as follows: (1) the negative group, rats treated with olive oil were transfused with PBS after 3, 4 and 5 wk of modeling; (2) the positive control group, rats treated with CCl₄ were transfused with PBS after 3, 4 and 5 wk; (3) the BDHSCs group, rats treated with CCl₄ were transfused with PBS containing BDHSCs after 3, 4 and 5 wk of CCl₄ induction; (4) the BM-EPCs group, rats treated with CCl₄ were transfused with PBS containing BM-EPCs after 3, 4 and 5 wk; and (5) the BM-EPCs/BDHSCs group, rats treated with CCl₄ were transfused with PBS containing BDHSCs after 4 wk of induction, and BM-EPCs after 3 and 5 wk. The transfusion after 4 wk of induction was 2×10^5 cells *via* the branch of portal vein, and transfusion after 3 or 5 wk was 2×10^6 cells *via* the tail vein. Rats were sacrificed after 6

wk of CCl₄ induction. The blood samples were collected from celiac artery for immediate biochemical detection. The liver tissues were stored at -80 °C or fixed in 10% formaldehyde for future analysis.

Masson's trichrome collagen staining of the liver

The liver tissues fixed with formaldehyde were embedded in paraffin, and then stained with Masson's trichrome collagen staining. The stages of liver fibrosis were graded by the semiquantitative staging scores as follows: 0, no collagen fibers; 1, slight fibrosis, collagen fibers located in the central liver lobule; 2, moderate fibrosis, widened central collagen fibers; 3, severe fibrosis, collagen fibers extended to the edge of liver lobule; 4, liver cirrhosis, pseudolobuli formation^[23].

Immunohistochemistry for VEGF and proliferating cell nuclear antigen (PCNA) in the liver

The liver tissue paraffin-embedded sections were blocked with 0.3% H₂O₂ in methanol for endogenous peroxidase activity, incubated with mouse anti-rat VEGF or PCNA monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, United States) and peroxidase-conjugated rabbit anti-mouse IgG (Santa Cruz Biotechnology). The sections were stained with diaminobenzidine and counterstained with hematoxylin. Quantification of PCNA expression was carried out by measuring the integrated optical density (IOD) of positive staining area using the ImagePro plus

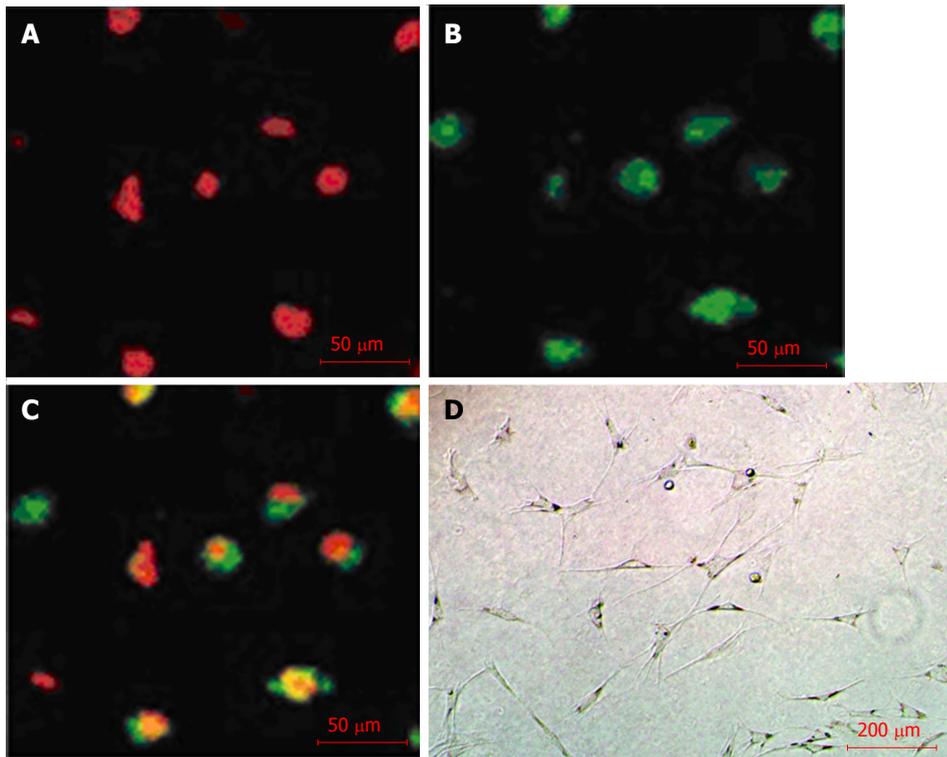


Figure 2 Phagocytosis and vasculogenesis functions of BM-EPCs of liver fibrosis rats. A: Bone marrow-derived endothelial progenitor cells uptake of Dil-ac-LDL (red); B: BM-EPCs binding with FITC-UEA-1 (green); C: Merge of A and B (yellow); D: Vascular network-like structures of BM-EPCs on Matrigel. Bars: 50 μm (A-C); 200 μm (D); BM-EPCs: Bone marrow-derived endothelial progenitor cells.

Table 1 Semiquantitative staging scores of liver fibrosis by Masson staining

| Group | n | Stage, n | | | | | Score |
|----------------|---|----------|---|---|---|---|------------------------------------|
| | | 0 | 1 | 2 | 3 | 4 | |
| Normal | 8 | 8 | 0 | 0 | 0 | 0 | 0 |
| Model | 8 | 0 | 0 | 1 | 6 | 1 | 3.00 \pm 0.19 ^a |
| BDHSCs | 8 | 0 | 0 | 2 | 5 | 1 | 2.88 \pm 0.23 ^a |
| BM-EPCs | 8 | 0 | 0 | 2 | 6 | 0 | 2.75 \pm 0.16 ^a |
| BM-EPCs/BDHSCs | 8 | 0 | 3 | 4 | 1 | 0 | 1.75 \pm 0.25 ^{a,c,e,g} |

Scores are presented as mean \pm SD. ^a $P < 0.05$ vs normal group; ^c $P < 0.05$ vs model group; ^e $P < 0.05$ vs BDHSCs group; ^g $P < 0.05$ vs BM-EPCs group.

image analysis software. Three random areas were selected per slide from two slides per sample.

Enzyme-linked immunosorbent assay for VEGF in the liver

The wet liver tissues (100 mg per sample) were homogenized in 1 mL PBS in the presence of 1% protease inhibitors (Sigma-Aldrich). The supernatant fractions of liver homogenates were used to measure the VEGF levels according to the manufacturer's instructions using Rat VEGF Quantikine Enzyme-linked immunosorbent assay (ELISA) Kits (R&D Systems, Minneapolis, MN, United States).

Real-time quantitative reversely transcribed polymerase chain reaction for hepatocyte growth factor in the liver

The extracted total RNA of liver tissues was reverse transcribed into cDNA using ExScriptTM RT reagent

Kit (Takara, Kusatsu, Japan). Real-time quantitative polymerase chain reaction (PCR) was performed using SYBR green II (Takara) on a Light Cycler (Roche Diagnostics GmbH, Penzberg, Germany). The forward and reverse primers were as follows: HGF (NM_017017, 122 bp), 5'-TTTCCCGTTGTGAAGGAGAT-3' and 5'-CCCTACTGTTGTTTGTGTTGGA-3'; GAPDH (NM_017008, 92 bp), 5'-GACATGCCGCCTGGAGAAAC-3' and 5'-AGCCCAGGATGCCCTTTAGT-3'. Real-time PCR was performed at 95 $^{\circ}\text{C}$ for 10 min, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 10 s, 60 $^{\circ}\text{C}$ for 60 s. To ensure specific amplification, a melting curve was generated at the end of the PCR for each sample. Hepatocyte growth factor (HGF) mRNA quantities were determined by comparative CT method by the Light Cycler software.

Liver biochemical assays in the blood

The biochemical indices of liver function, including alanine aminotransferase aspartate (ALT), aspartate aminotransferase (AST) and total bilirubin (TBIL) in serum, and prothrombin time (PT) and activated partial thromboplastin time (APTT) in plasma, were detected by using an automated analyzer (LX20; Beckman Coulter, Brea, CA, United States).

Statistical analysis

All data were presented as means \pm SD and analyzed using SPSS 17.0 statistical software. The differences between the mean values of each group were compared by the one-way analysis of variance (ANOVA) or Kruskal-Wallis test as appropriate, and considered to

Table 2 Biochemical indexes of liver function in the blood

| Group | <i>n</i> | ALT, IU/L | AST, IU/L | TBIL, μ mol/L | PT, s | APTT, s |
|----------------|----------|-------------------------------|--------------------------------|------------------------------|-------------------------------|-----------------------------|
| Normal | 8 | 62.5 \pm 12.7 | 153.5 \pm 13.6 | 6.6 \pm 0.3 | 16.6 \pm 1.0 | 21.9 \pm 1.5 |
| Model | 8 | 144.8 \pm 61.5 ^a | 269.6 \pm 83.3 ^a | 17.3 \pm 2.6 ^a | 20.5 \pm 1.1 ^a | 26.2 \pm 2.8 ^a |
| BDHSCs | 8 | 70.4 \pm 19.5 ^c | 260.4 \pm 84.2 ^a | 13.5 \pm 1.8 ^{ac} | 19.5 \pm 1.3 ^a | 20.1 \pm 2.7 ^c |
| BM-EPCs | 8 | 65.8 \pm 26.5 ^c | 159.3 \pm 41.3 ^c | 14.3 \pm 2.3 ^{ac} | 19.4 \pm 1.4 ^a | 19.9 \pm 2.2 ^c |
| BM-EPCs/BDHSCs | 8 | 61.6 \pm 13.3 ^c | 135.0 \pm 33.3 ^{cc} | 6.5 \pm 1.1 ^{ceg} | 16.6 \pm 1.0 ^{ceg} | 19.8 \pm 2.3 ^c |

Data are presented as mean \pm SD. ^a*P* < 0.05 vs normal group; ^c*P* < 0.05 vs model group; ^a*P* < 0.05 vs BDHSCs group; ^{ac}*P* < 0.05 vs BM-EPCs group.

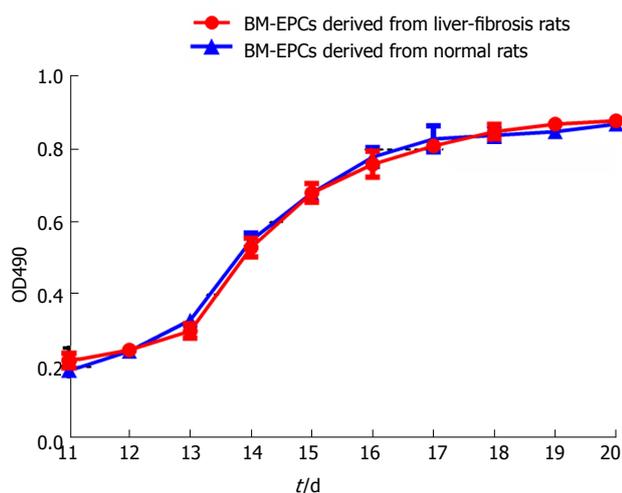


Figure 3 Growth curves of BM-EPCs. Between days 11 and 20 of culture induction, the growth curve (OD490 values) of BM-EPCs of liver fibrosis rats (red) was similar to that of BM-EPCs of normal rats (blue); BM-EPCs: Bone marrow-derived endothelial progenitor cells.

be statistically significant when the adjusted *P* values were < 0.05 (two-tailed).

RESULTS

Phenotype and purity of BM-EPCs

After 4 d of culture inductions, BM-EPCs were adherent (Figure 1A). After 10 d, BM-EPCs formed colonies (Figure 1B), and expression of CD133 and VEGFR2 were detected (Figure 1E). The co-expression level was 63.9% \pm 2.15%, which was significantly higher than that of cells cultured without induction (37.2% \pm 1.35%, *P* < 0.05) (Figure 1D).

Phagocytosis and vasculogenesis functions of BM-EPCs

After 10 d of culture induction, uptake of Dil-ac-LDL (red in Figure 2A) and binding with FTIC-UEA-1 (green in Figure 2B) to BM-EPCs were observed. The cells with yellow double-fluorescence were differentiating EPCs (Figure 2C). Furthermore, BM-EPCs formed a stable vascular network-like structure on Matrigel (Figure 2D).

Cytokinetics of BM-EPCs and localization of BM-EPCs in the liver

The growth curve of BM-EPCs from rats with liver fibrosis was similar to that of BM-EPCs of normal rats from day 11 to day 20 post induction. The logarithmic

growth phase of cells was 3-7 d. The cells grew slowly and plateaued at day 18 (Figure 3). We implanted BM-EPCs in rats with fibrotic liver. We did not detect significant red fluorescence in the fibrotic liver tissue without implantation. BM-EPCs labeled with PKH26 were implanted in the fibrotic liver, and BM-EPCs were located primarily in/near hepatic sinusoids as indicated by nuclear staining with DAPI (data not shown).

Location of BM-EPCs in the liver

BM-EPCs labeled with PKH26 implanted in the fibrotic liver (red in Figure 4B). The DAPI nuclear staining result showed that BM-EPCs were located primarily in/near hepatic sinusoids (Figure 4C).

Collagen formation and improvement of liver fibrosis degree

Masson staining results showed there were significant numbers of collagen fibers (green) formed in the liver tissue of liver fibrosis rats in comparison to that in normal liver tissue (Figure 5A and B), and the staging score of liver fibrosis was 3.00 \pm 0.19, which was dramatically higher than that in normal rats (0, *P* < 0.05). Transplantations of BDHSCs or BM-EPCs alone and combination of both suppressed the formation of collagen fibers (Figure 5C-E). There was no significant difference between the staging scores of liver fibrosis in the BDHSCs (2.88 \pm 0.23) or BM-EPCs (2.75 \pm 0.16) groups and that of the model group with only CCl₄ treatment (*P* > 0.05). However, transplantation of BM-EPCs/BDHSCs combination, significantly reduced the degree of liver fibrosis (staging score of 1.75 \pm 0.25, *P* < 0.05) (Table 1).

Increase of VEGF levels in the liver

Immunohistochemistry (Figure 6A-E) and ELISA (Figure 6F) results showed that VEGF levels in the liver tissues in the model group with CCl₄ treatment alone were higher than that in the normal group (440.95 pg/mL \pm 65.11 pg/mL vs 349.70 pg/mL \pm 56.50 pg/mL per 100 mg liver tissue, *P* < 0.05). There were no significant differences between those in the model group and the BDHSCs group (461.28 \pm 23.78, *P* > 0.05). The VEGF levels were increased in the BM-EPCs (707.10 \pm 54.32) and BM-EPCs/BDHSCs group (615.42 \pm 42.96), compared with those in the model group and BDHSCs group (*P* < 0.05). BM-EPCs transplantation slightly increased VEGF level but not significantly, as compared with that of combined transplantation of

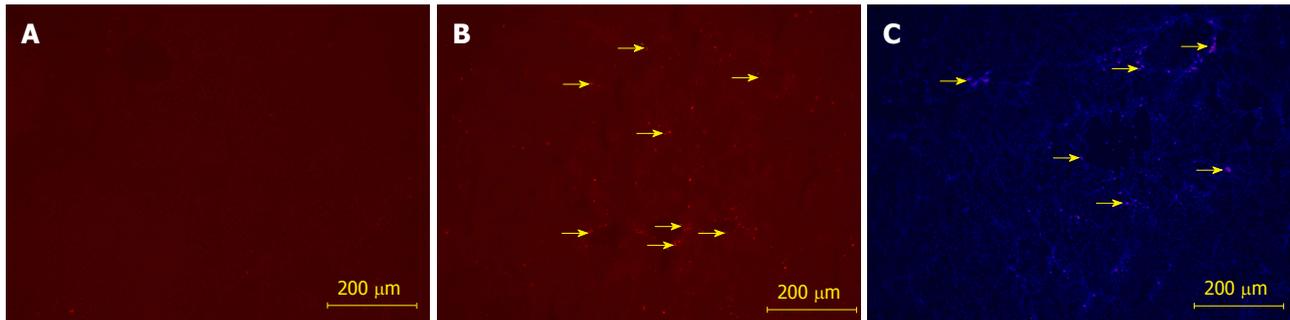


Figure 4 Location of BM-EPCs of liver fibrosis rats in the fibrotic liver. A: Autofluorescence of the fibrotic liver tissue; B: BM-EPCs labeled with PKH26 (red) implanted in the fibrotic liver tissue (yellow arrow); C: BM-EPCs (red) located in/near hepatic sinusoids (yellow arrow) on the background of DAPI staining (blue). Bars: 200 µm; BM-EPCs: Bone marrow-derived endothelial progenitor cells.

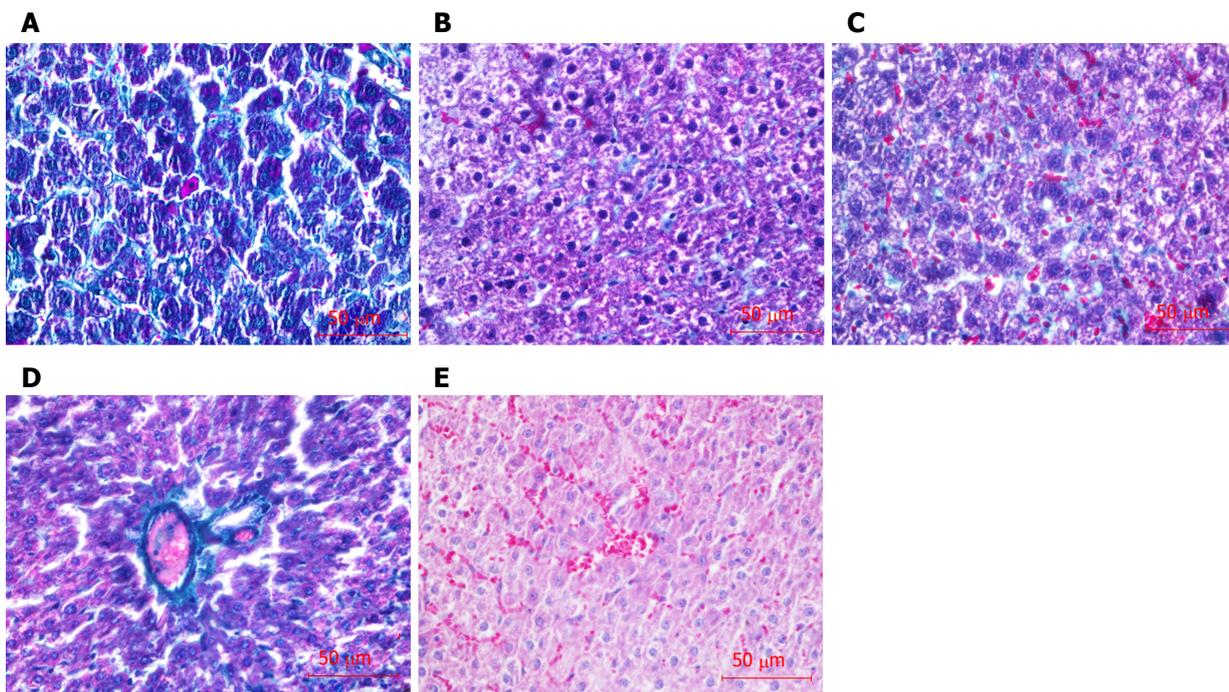


Figure 5 Collagen formation in the liver shown by masson staining (green). A: Normal group; B: Model group; C: Bone marrow-derived hepatocyte stem cells group; D: Bone marrow-derived endothelial progenitor cells group (BM-EPCs); E: BM-EPCs/BDHSCs group. Bars: 50 µm.

BM-EPCs and BDHSCs ($P > 0.05$).

Enhancement of liver regeneration

We evaluated liver regeneration after transplantation by detecting the expressions of PCNA protein and HGF gene in the liver. We found that compared with those in the normal group, the expressions of PCNA protein (Figure 7A-F) and HGF mRNA (Figure 8) in the model group was slightly but not significantly increased ($P > 0.05$). Significant increases in the levels of PCNA protein and HGF mRNA were observed in the BDHSCs, BM-EPCs and BM-EPCs/BDHSCs compared with those in the model group ($P < 0.05$), and combination of BM-EPCs/BDHSCs transplantation induced maximal up-regulation of PCNA protein and HGF mRNA levels.

Improvement of liver function

As shown in Table 2, the levels of ALT, AST, TBIL, PT and APTT were increased in the model group, compared with those in the normal group ($P < 0.05$).

Compared with the model group, transplantation of BDHSCs reduced the levels of ALT, TBIL and APTT ($P < 0.05$), but failed to improve the levels of AST and PT ($P > 0.05$). Furthermore, levels of ALT, AST and APTT in the group with BM-EPCs transplantation were lower than that in the model group ($P < 0.05$), but there was no significant difference in levels of TBIL and PT between the two groups. The levels of ALT, AST, TBIL, PT and APTT in the BM-EPCs/BDHSCs group were significantly improved, to be equivalent to normal levels ($P > 0.05$) compared with those in the BDHSC (AST, TBIL and PT, $P < 0.05$) and BM-EPCs (TBIL and PT, $P < 0.05$) groups (Table 2).

DISCUSSION

Hepatic neovascularization and endothelial/sinusoidal remodeling are critical for the treatment of liver fibrosis, in addition to liver regeneration^[24,25], and EPCs have been shown to play this role in liver fibrosis^[10,13]. The

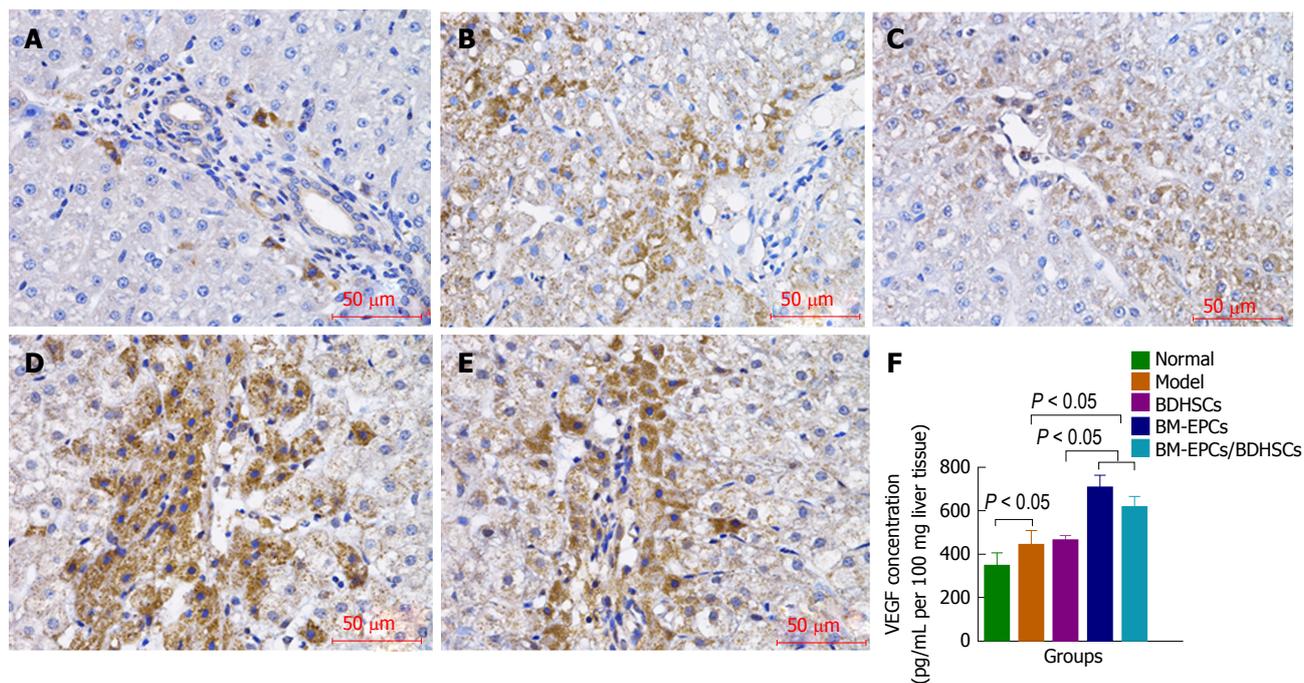


Figure 6 Vascular endothelial growth factor levels in the liver. A-E: VEGF protein expressions shown by immunohistochemistry (brown). A: Normal group; B: Model group; C: BDHSCs group; D: BM-EPCs group; E: BM-EPCs/BDHSCs group; F: VEGF concentration detected by ELISA. $n = 8$; VEGF: Vascular endothelial growth factor; BM-EPCs: Bone marrow-derived endothelial progenitor cells; BDHSCs: Bone marrow-derived hepatocyte stem cells.

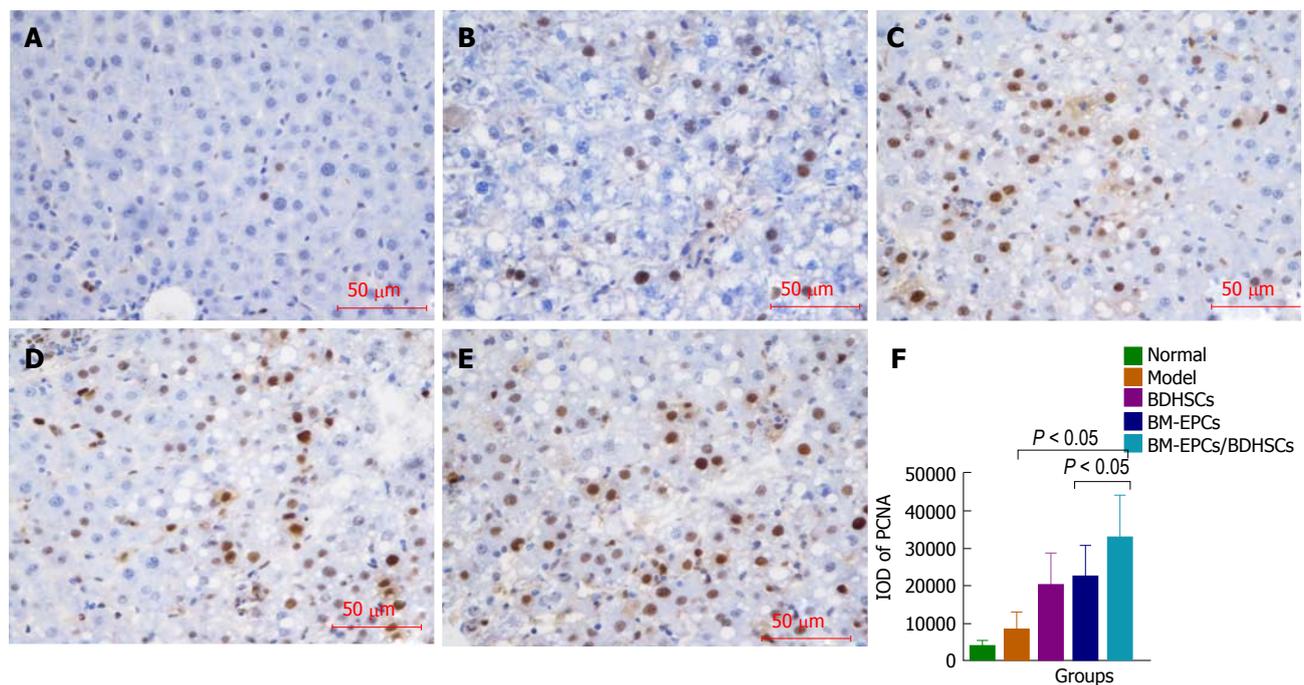


Figure 7 Proliferating cell nuclear antigen protein expression shown by immunohistochemistry (brown). A: Normal group; B: Model group; C: BDHSCs group; D: BM-EPCs group; E: BM-EPCs/BDHSCs group; F: Quantitative analysis of PCNA expression by IOD. ($n = 8$); BM-EPCs: Bone marrow-derived endothelial progenitor cells; BDHSCs: Bone marrow-derived hepatocyte stem cells.

majority of EPCs exist in bone marrow and peripheral blood. EPCs are rare in blood, since their proportion is only 0.005%-0.01% of that of white blood cells in blood^[26]. Bone marrow contains numerous hematopoietic stem cells and mesenchymal stem cells, which can differentiate into EPCs^[27]. Therefore, bone marrow may be the major source of EPCs in

potential clinical treatment of liver fibrosis. BM-EPCs used in recent therapeutic studies were derived from healthy individuals^[11-15]. Thus, the clinical application of EPCs transplantation is limited, due to rare EPCs in peripheral blood and shortage of normal allogeneic bone marrow.

Whether sufficient normal EPCs can be obtained from

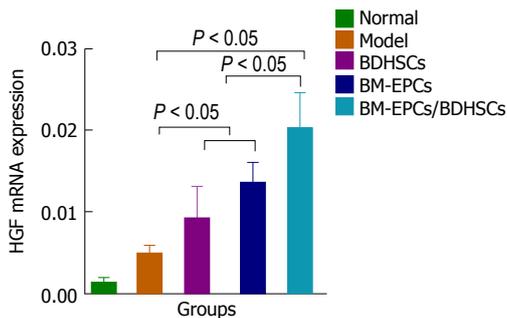


Figure 8 Hepatocyte growth factor mRNA expression detected by real-time quantitative polymerase chain reaction. $n = 8$; BM-EPCs: Bone marrow-derived endothelial progenitor cells; BDHSCs: Bone marrow-derived hepatocyte stem cells; HGF: Hepatocyte growth factor.

bone marrow cells in liver fibrosis pathoenvironment is a critical issue for the clinical application of autogenous EPCs therapy. A recent study showed that the functional homeostasis of bone marrow-derived $c\text{-Kit}^+$, Sca-1^+ and Lin^- (BM-KSL) cells, as a stem cell fraction of BM-EPCs, was disrupted in liver fibrosis, indicating a weakened regenerative capability and decreased differentiation potential in liver fibrosis mice^[28]. It is predicted that disturbed EPC differentiation might give rise to inadequate production for angiogenesis and tissue regeneration^[28].

Whether normal EPCs can be obtained by *in vitro* culture from bone marrow in liver fibrosis rats remains uncertain. In the present study, abundant EPCs that possess specific phenotypes (VEGFR2 and CD133) and functions (phagocytosis and vasculogenesis) as well as normal proliferation and differentiation abilities were successfully obtained from bone marrow in liver fibrosis rats by collection and culture induction of plastic-nonadherent cells *in vitro*. EPCs do not easily attach on the bottom of surface of a culture plate, and thus different-rate adherent culture is a way to obtain EPC-differentiated cells^[27,28]. BM-KSL cells are only a small portion of plastic-nonadherent cells^[29]. Therefore, only some of the BMSCs in the liver fibrosis pathoenvironment might be hypophrenic. These hypophrenic cells in BMSCs could be eliminated automatically in the process of different-speed adherent and culture induction *in vitro*.

In the present study, transplanted BM-EPCs were located primarily in/near hepatic sinusoids, suggesting in the *in vivo* pathoenvironment of liver fibrosis, exogenous BM-EPCs derived from liver fibrosis rats had the ability to reside at sites of vessels in the liver.

In order to achieve the dual effects of hepatic neovascularization and liver regeneration, we next compared the effects of transplantations of BM-EPCs, BDHSCs and the combination of both on liver fibrosis. The increased levels of VEGF were observed in both BM-EPCs and BM-EPCs/BDHSCs groups, but not in the BDHSCs group. It has been reported that EPCs release vasoactive substances that promote angiogenesis

by autocrine and paracrine signaling, such as VEGF, EGF and platelet-derived growth factor^[11,14]. Among them, VEGF plays an essential role in both the neovascularization and the enhancement of sinusoidal density in the liver^[27]. Studies have shown that the release of VEGF is essential for permeabilization of liver sinusoidal endothelial cells and integration of transplanted cells in the liver parenchyma^[30,31]. Conversely, this study has shown that the effect of BM-EPCs transplantation on promoting VEGF secretion was slightly stronger than that of combined transplantation of BM-EPCs and BDHSCs. This result suggested that the release of VEGF in the liver might be controlled mainly by EPCs, but not BDHSCs.

Furthermore, an enhancement of liver regeneration manifested by increased HGF levels was observed in the rats with combined transplantation of BM-EPCs and BDHSCs^[32], and this effect was greater than that of transplantation of BDHSCs or BM-EPCs alone. We deduced that BDHSCs might promote self-proliferation and mobilize endogenous liver regeneration through secretion of growth factors such as HGF^[33,34], and EPCs can restore the permeabilization of the endothelial barrier interposed between liver sinusoids and parenchyma by releasing vasoactive substances and vasodilator-related molecules^[30,31].

The integral effect of BM-EPCs transplantation on suppression of hepatic fibrogenesis and improvement of liver function was similar to that of BDHSCs transplantation. However, the combined effect of transplantation of BM-EPCs and BDHSCs exceeded individual transplantations significantly.

In summary, our study demonstrated that it was feasible to obtain normal EPCs from bone marrow in liver fibrosis rats and that combined transplantation of BM-EPCs and BDHSCs are effective for treating liver fibrosis. These findings highlight the clinical value of EPCs autotransplantation and the potential application of a novel strategy of therapy for liver fibrosis that involves combined transplantation of BM-EPCs and BDHSCs. Further studies are need to be carried out to explore the reciprocal mechanism of BM-EPCs and BDHSCs in liver fibrosis. In addition, whether BM-EPCs might promote tumor angiogenesis and cause potential tumor growth^[2] in the process of treating liver fibrosis should also be evaluated in further study.

ARTICLE HIGHLIGHTS

Research background

Hepatic neovascularization and endothelial/sinusoidal remodeling are critical for the treatment of liver fibrosis, in addition to liver regeneration. Transplantation of bone marrow-derived endothelial progenitor cells (BM-EPCs) has potential roles in hepatic neovascularization, endothelial/sinusoidal remodeling and increased hepatic blood flow for the treatment of liver fibrosis. However, BM-EPCs were all derived from healthy rats in these studies. We sought to evaluate the feasibility of clinical application of autogenous EPCs therapy by using normal BM-EPCs obtained from liver fibrosis rats, and evaluated the effectiveness of combined transplantation of BM-EPCs and BDHSCs *in vivo* for the treatment of

liver fibrosis.

Research motivation

The author observed the feasibility of obtaining normal BM-EPCs from the liver fibrosis environment in rats. We evaluated the effectiveness for treating liver fibrosis by combined transplantation of BM-EPCs and bone marrow-derived hepatocyte stem cells (BDHSCs) in order to achieve the dual effects of hepatic neovascularization and liver regeneration. These findings highlight the clinical value of EPCs autotransplantation, and potential application of a novel strategy of therapy for liver fibrosis that involves combined transplantation of BM-EPCs and BDHSCs.

Research objectives

Our research objectives were to explore the feasibility of obtaining normal BM-EPCs from the liver fibrosis environment and the effectiveness for treating liver fibrosis by combined transplantation of BM-EPCs and BDHSCs. The significance of realizing these objectives were to highlight the clinical value of EPCs autotransplantation and potential application of a novel strategy of therapy for liver fibrosis that involves combined transplantation of BM-EPCs and BDHSCs.

Research methods

For the treatment of liver fibrosis, BDHSCs, which can promote hepatic cell regeneration, combined with BM-EPCs, which can promote hepatic revascularization, were transplanted into rats with liver fibrosis. The normal functional BM-EPCs and BDHSCs were both successfully obtained from liver fibrosis rats. Tracing analysis was conducted by labeling EPCs with PKH26 *in vitro* to show EPCs' location in the liver. After transplantation of BM-EPCs, BDHSCs or both into liver fibrosis rats, the indicators of hepatic neovascularization, liver regeneration, collagen formation, liver fibrosis degree and liver function were observed and detected.

Research results

Normal functional BM-EPCs from liver fibrosis rats were successfully obtained. The co-expression level of CD133 and VEGFR2 was $63.9\% \pm 2.15\%$. Transplanted BM-EPCs were located primarily in/near hepatic sinusoids. The combined transplantation of BM-EPCs and BDHSCs promoted hepatic neovascularization, liver regeneration and liver function, and decreased collagen formation and liver fibrosis degree.

Research conclusions

This study demonstrated that it was feasible to obtain normal BM-EPCs "filtered" by *in vivo* pathological environment from bone marrow of a liver fibrosis rat model. The combined transplantation of BM-EPCs and BDHSCs was performed in order to achieve the dual effects of hepatic neovascularization and liver regeneration for liver fibrosis. The combined transplantation exhibited maximal therapeutic effect compared to that of transplantation of BM-EPCs or BDHSCs alone. These findings highlight the clinical value of EPCs autotransplantation and the potential application of a novel strategy of therapy for liver fibrosis that involves combined transplantation of BM-EPCs and BDHSCs.

Research perspectives

Our research is still at the stage of animal experiments. Further studies are needed to explore the reciprocal mechanism of BM-EPCs and BDHSCs in liver fibrosis. Clinical experiments should be carry out gradually. In addition, whether BM-EPCs might promote tumor angiogenesis and cause potential tumor growth in the process of treating liver fibrosis should also be evaluated in further study.

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