Original Article

Bone Marrow Cells Ameliorate Liver Fibrosis and Express Albumin after Transplantation in CCl_4-Induced Fibrotic Liver

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ABSTRACT

Background/Aim: We investigated the effect of bone marrow–derived stem cell (BMSC) transplantation on carbon tetrachloride (CCl_4)-induced liver fibrosis. **Patients and Methods:** BMSCs of green fluorescent protein (GFP) mice were transplanted into 4-week CCl_4-treated C57BL/6 mice directly to the liver, and the mice were treated for 4 more weeks with CCl_4 (total, 8 weeks). After sacrificing the animals, quantitative data of percentage fibrosis area and the number of cells expressing albumin was obtained. One-way analysis of variance was applied to calculate the significance of the data. **Results:** GFP expressing cells clearly indicated migrated BMSCs with strong expression of albumin after 28 days post-transplantation shown by anti-albumin antibody. Double fluorescent immunohistochemistry showed reduced expression of αSMA on GFP-positive cells. Four weeks after BMSC transplantation, mice had significantly reduced liver fibrosis as compared with that of mice treated with CCl_4 assessed by Sirius red staining. **Conclusion:** Mice with BMSC transplantation with continuous CCl_4 injection had reduced liver fibrosis and a significantly improved expression of albumin compared with mice treated with CCl_4 alone. These findings strengthen the concept of cellular therapy in liver fibrosis.

Key Words: Albumin, green fluorescent protein (GFP), liver fibrosis, mice, αSmooth Muscle Actin (α SMA)

Received: 12.12.2011, Accepted: 06.03.2012
How to cite this article: Ali G, Masoud MS. Bone marrow cells ameliorate liver fibrosis and express albumin after transplantation in CCl 4 -induced fibrotic liver. Saudi J Gastroenterol 2012;18:263-7.

Liver fibrosis is characterized as the repeated injury causing the remodeling of the liver tissue resulting in the excessive accumulation of extracellular matrix with scar tissue encapsulating the area of injury.[1] This condition resulted in many clinical complications, such as ascites, variceal hemorrhage, and encephalopathy.[2] Different diseases, such as Wilson's disease, viral hepatitis B and C (HBV and HCV), liver cancer, and cirrhosis can affect the liver. Carbon tetrachloride (CCl_4) has been widely used to induce liver fibrosis in experimental animals. The mechanism involves the addition of one electron to CCl_4 on binding to ferric cytochrome P450 and cleaving of the carbon–chloride bond. This binding results in a highly reactive trichloromethyl free radical and causes liver cell necrosis and destruction of the extracellular matrix through lipid peroxidation of membranes.[3-5]

Access this article online
Quick Response Code:
Website: www.saudijgastro.com
DOI: 10.4103/1319-3767.98433

The nonhematopoietic part of the bone marrow, referred to as bone marrow-derived mesenchymal stem cells (BMSCs), are of interest because of the simple isolation from a small aspirate of bone marrow.[6] BMSCs have shown a large *in vitro* and *in vivo* differentiation potential.[7] Their hepatic potential was first described by Lee *et al*.[8] Some recent studies also evidenced *in vivo* potential of BMSCs.[9] A key advantage of using BMSCs is their immunologic properties.[10] They were first shown to be engrafted in the recipient's liver and differentiated into hepatocytes by Petersen *et al*.[11] Therefore, BMSCs can be a useful source for cellular therapy in treating liver fibrosis.[12]

BMSCs could repair CCl_4-injured liver by reducing inflammation, collagen deposition, and remodeling.[13] Sakaida *et al*[14] found that BMSCs treatment to 4-week CCl_4-induced rats would result in significantly reduced liver fibrosis. A study by Oh *et al*[15] found that two of the liver-specific proteins alfa-feto protein and albumin were expressed in rat bone marrow cell culture. Some other studies also reported that BMSCs can express albumin in *in vitro* conditions.[16-18] In this present study, green fluorescent protein $(GFP)^+$ BMSCs were transplanted into CCl_4-induced liver fibrotic mouse model. The co-expression of albumin in the GFP^+

BMSCs was observed in CCl_4-induced liver after 2 and 4 weeks of BMSCs transplantation. After 4 weeks, transplanted GFP^+ BMSCs showed increased expression of albumin and a significant reduction in fibrosis was observed.

PATIENTS AND METHODS

Mice

GFP-transgenic mice and C57BL/6 wild-type mice were used in the experiment. All animals were treated according to procedures approved by the Institutional Review Board at the National Center of Excellence in Molecular Biology, Lahore, Pakistan. All animals were housed in conventional cages under controlled conditions of temperature (23°C ± 3°C) and relative humidity (50% ± 20%), with light illumination for 12 h/day.

BMSCs preparation

For BMSCs isolation, GFP-transgenic mice (6 weeks old) were killed and the limbs were removed. BMSCs were flushed with Dulbecco's Modified Eagle Medium (DMEM) from the medullary cavities of tibias and femurs using a 25-G needle. Then cells were cultured in DMEM medium supplement with 20% FBS (Sigma, USA), 100 μg/mL streptomycin and 100 U/mL penicillin in a 25 cm^2 culture flasks and incubated at 37°C in an atmosphere containing 5% CO_2. When cell confluency reached to 70–80%, they were detached with 0.25% Trypsin and 0.1% EDTA, and subcultured in two 25 cm^2 flasks till second passage.

Experimental protocol

Six weeks old female C57BL/6 mice were treated with 1 ml/kg CCl_4 dissolved in olive oil (1:1) twice a week for 4 weeks intraperitoneally.[14] Animals were divided into 3 groups (n = 10 each); CCl_4 control group, BMSCs transplanted group sacrificed after 2 weeks and BMSCs transplanted group sacrificed after 4 weeks. Twenty-four hours after the last injection of CCl_4, 1×10^5 GFP^+ BMSCs were injected directly into liver as described previously.[19] Same volume of saline water was injected in CCl_4 control animals. After 2 and 4 weeks of post-transplantation, mice were sacrificed to assess the extent of liver fibrosis and expression of albumin. All mice were kept on receiving CCl_4 during the post-transplantation period.

Histology of liver tissue

The liver was perfused via the heart with 4% paraformaldehyde to flush out blood cells and incubated with 4% paraformaldehyde overnight. Tissues were then soaked in 30% sucrose for 3 h and frozen in liquid nitrogen to prepare for sectioning. Cells expressing albumin and αSMA were analyzed by immunohistochemistry using anti-albumin (1:50; Abcam, USA) and anti-αSMA antibodies (1:400; Sigma–Aldrich, Germany). Tissues were soaked in 0.3% Triton X-100 with 2% normal goat serum (Chemi-Con, Temecula, CA, USA). Sections were incubated with primary antibodies overnight at 4°C. For fluorescent immunohistochemistry, we used fluorescein isothiocyanate–conjugated anti-mouse as secondary antibody. For the evaluation of fibrosis, picro-sirius red staining was performed using 0.1% picro-sirius red solution as previously described. Fluorescence images were taken by Olympus BX61 microscope equipped with DP-70 camera (Olympus, Japan).

Quantitative analysis of liver fibrosis

We quantified the liver fibrosis area with picro-sirius red staining using an Olympus microscope equipped with a DP-70 camera (Tokyo, Japan). Briefly, the red area, considered the fibrotic area, was assessed by computer-assisted image analysis with Image J software (NIH). The mean value of 3 randomly selected areas per sample was used as the expressed percent area of fibrosis.

Gene expression analysis

RNA from liver tissue of all experimental groups was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using 1μg of total RNA by Revert Aid H Minus first strand cDNA synthesis kit (Fermentas, Glen Burnie, Maryland, USA). Gene-specific primers were designed using online software Primer3 (http://frodo.wi.mit.edu/primer3/). Sequences of the genes were taken from NCBI. All primer sequences are mentioned in Table 1 and following thermal conditions: 4 min at 94°C, followed by 31–35 cycles of 94°C for 45 s, 56–58°C for 45 s, and 72°C for 45 s, then final extension at 72°C for 10 min. PCR products were visualized and photographed after electrophoresis in 2% (w/v) agarose gels containing 0.5 mg/mL ethidium bromide.

Statistical analysis

Quantitative data of percent fibrosis area and cells expressing albumin of 10 animals was obtained. Results are presented as the mean ± SD. Differences between groups were analyzed by One-way analysis of variance.

RESULTS

Mice chronically infected with CCl_4 were treated with GFP^+ BMSCs by injecting into the left lateral lobe of the liver. Bright GFP^+ cells were engrafted and observed in all lobes of the liver, indicating cell migration from left lateral lobe to other injury sites as well [Figure 1b] as compared to non-transplanted mice [Figure 1a]. GFP^+ cells in mice sacrificed after 2 weeks co-expressed albumin in the hepatic parenchyma [Figure 2b]. Later at 4th week, the transplanted sections expressed GFP^+ cells with enhanced expression of albumin [Figure 2c]. GFP^+ albumin expressing cells were not present in the

Table 1: Primer sequences

Primers	Forward	Reverse
Collagen Iαl	GTGGACCTCCTGGACCTCAG	AGGAGCTCCGTTTTCACCAG
αSMA	CTGACAGAGGCACCACTGAA	CAGAGGCATAGAGGGACAGC
Albumin	TTAGTGAGGTGGAGCATGAC	GTCTCAGCAACAGGGATACA
MMP-9	AAGGCAAACCCTGTGTGTTC	CCAGGGATGGACCAAACTAA
β-Actin	ACTGCTCTGGCTCCTAGCAC	ACATCTGCTGGAAGGTGGAC

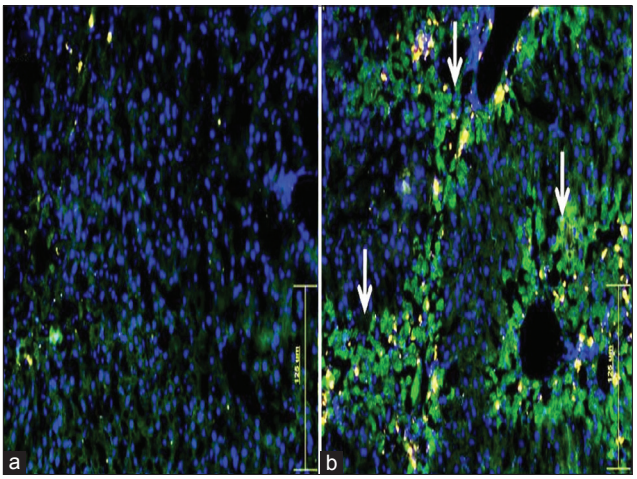

Figure 1: Homing of transplanted GFP+ BMSCs in CCl_4 (a) and BMSCs (b) groups. DAPI was used to identify nuclei (magnification = 200×; *n* = 10 each)

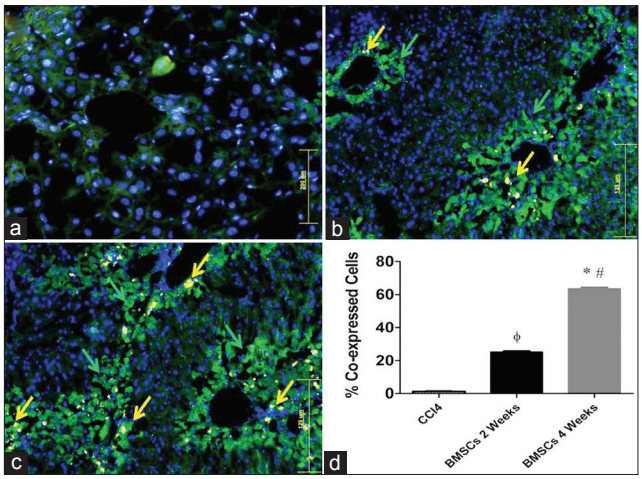

Figure 2: Immunohistochemical expression of albumin in mouse liver. (a) CCl_4 group, (b) BMSCs transplanted group sacrificed after 2 weeks, (c) BMSCs transplanted group sacrificed after 4 weeks (original magnification = 200×), (d) one-way analysis of variance was applied to check the significance of the data (*n* = 10). All values are expressed as mean ± SEM. $^{*}P < 0.05$ for BMSCs 4 weeks vs CCl_4 control, $^{\#}P < 0.05$ for BMSCs 4 weeks vs BMSCs 2 weeks, $^{\Phi}P < 0.05$ for BMSCs 2 weeks vs CCl_4 control

fibrotic mouse liver transplanted with saline water [Figure 2a]. Statistical data [Figure 2d] showed that BMSCs transplanted liver showed significantly higher number of cells expressing albumin (60 cells/field) after 4 weeks of BMSCs transplantation as compared with other groups (22 cells/field in BMSCs 2 weeks group).

Fluorescence staining (anti-α smooth muscle actin with green color) indicated that a fine network pattern of stellate cells existed in the liver treated with CCl_4 alone [Figure 3a]. Conversely, GFP+ BMSCs transplanted groups showed reduced expression of αSMA in fibrotic liver [Figure 3b and c]. Mice sacrificed after 4 weeks of BMSCs transplantation showed significantly reduced expression of αSMA as compared with other groups [Figure 3c].

After 4 weeks, the BMSCs transplanted liver clearly showed reduction of liver fibrosis compared to the group treated with CCl_4 alone and BMSCs transplanted groups [Figure 4a–c]. Quantitative analysis [Figure 4d] of liver fibrosis by Image J software indicated that the percent fibrotic area of liver after 4 weeks of BMSCs transplantation was significantly reduced to 1.17% ± 0.34% as compared to other groups (2.35% ± 0.66 = BMSCs 2 weeks group and 4.56% ± 1.75 = CCl_4 group). The reverse transcriptase PCR (RT-PCR) analysis showed reduction in the expression of αSMA and collagen 1α1 genes after 4 weeks of BMSCs transplantation while the expression of these genes was increased after CCl_4 injury. MMP-9 is known to be an antifibrotic marker released by BMSCs.[20] After BMSCs transplantation the expression of MMP-9 was noted to increase along with albumin, which is a hepatic functional marker [Figure 5].

DISCUSSION

In this study, CCl_4-induced liver fibrosis was ameliorated after transplantation of BMSCs. BMSCs could ameliorate liver fibrosis by expressing certain antifibrotic factors, such as MMP-9[18] and also by releasing some factors, such as soluble Kit-ligand related to the differentiation and proliferation of transplanted BMSCs in liver inflammation induced by continuous injection of CCl_4.[14] BMSCs secrete certain growth factors, such as hepatic growth factor (HGF), nerve growth factor (NGF), and many cytokines. These have antiapoptotic activity in hepatocytes and play an essential part in the regeneration of liver.[21,22]

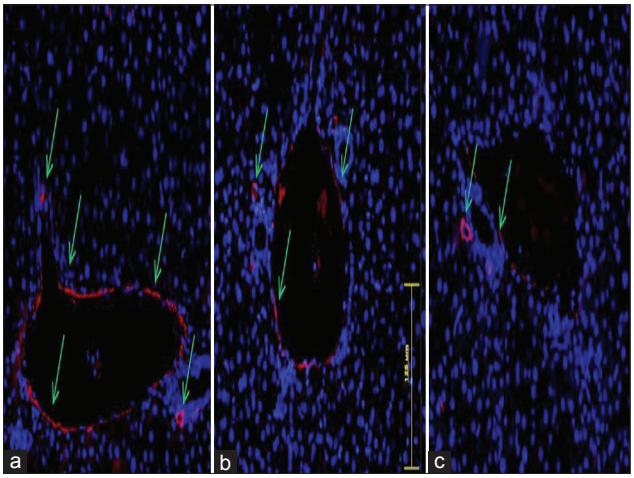

Figure 3: Immunohistochemical expression of αSMA in fibrotic mouse. (a) CCl_4 group, (b) BMSCs transplanted group sacrificed after 2 weeks (c) BMSCs transplanted group sacrificed after 4 weeks. Considerable reduction in the expression of αSMA can be observed (original magnification = 200×)

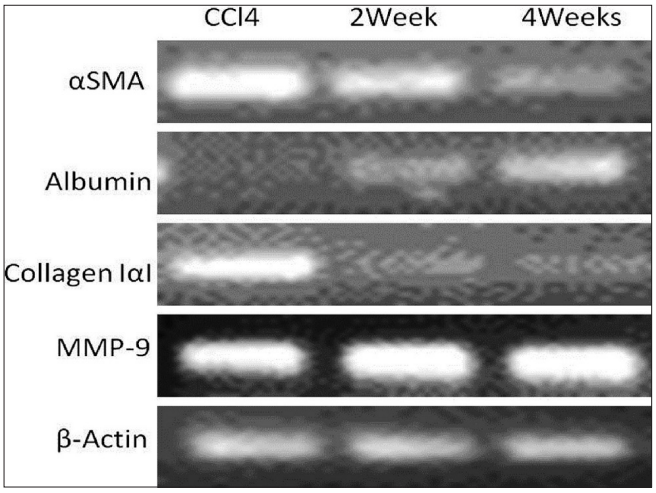

Figure 5: RT-PCR analysis of fibrotic (αSMA, collagen 1α1), antifibrotic (MMP-9), and hepatic (albumin) genes following the BMSCs transplantation in CCl_4-injured mouse liver after 2 and 4 weeks post-transplantation

MMP-9 was reported to have a role in the migration of BMSCs to the inflammatory site.[23] Transplanted BMSCs resulting in the degradation of the extracellular matrix may presumably lead to improved liver function and better survival of mice. According to our present data, increased expression of GFP^+ cells at the site of liver injury contributed to the degradation of interstitial collagens, which has been shown by cirius red staining [Figure 4]. This collagen is degraded to gelatin, which was degraded by MMP-9 resulting in the regression of fibrosis.[24] Our study indicated an enhanced expression of *MMP-9* [Figure 5] and reduced collagen deposition [Figure 4] after BMSCs transplantation.

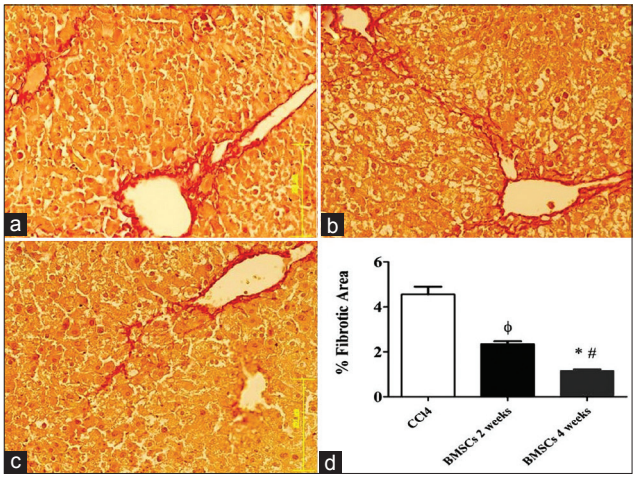

Figure 4: Photomicrographs of liver sections stained with sirius red. (a) CCl_4 group, (b) BMSCs transplanted group sacrificed after 2 weeks, (c) BMSCs transplanted group sacrificed after 4 weeks (original magnification = 200×), (d) quantitative analysis of fibrosis in different experimental groups. One-way analysis of variance was applied to check the significance of the data (n = 10 each group). All values are expressed as mean ± SEM. $^*P < 0.05$ for BMSCs 4 weeks vs CCl_4 control, $^{\#}P < 0.05$ for BMSCs 4 weeks vs BMSCs 2 weeks, $^{\Phi}P < 0.05$ for BMSCs 2 weeks vs CCl_4 control

The expression of αSMA, which is a marker of activated hepatic stellate cells, was reduced significantly after 4 weeks of GFP^+ BMSCs transplantation [Figures 3 and 5]. Thus, transplanted BMSCs may affect activated stellate cells by inhibiting them or by leading them to apoptosis.[25] Collagen 1α1, another fibrotic factor produced by activated hepatic stellate cells, was accumulated after CCl_4-induced fibrotic liver injury. Our results indicated a reduced .RNA expression of *collagen Iα1* gene after 4 weeks of BMSCs transplantation compared with the liver treated with CCl_4 alone. These results are in line with those of Sakaida *et al*'s.[14]

Fang *et al.*[13] reported that albumin-positive donor–derived cells were found at a lower frequency in CCl_4-injured liver tissue. Some other studies reported an increase in the serum albumin level.[26,27] In the current study, the expression of albumin was observed along with GFP^+ BMSCs, indicating that albumin is expressed by the transplanted BMSCs [Figures 1 and 2]. RT-PCR analysis also showed a marked increase in the expression of *albumin* after 4 weeks of BMSCs transplantation [Figure 5].

The present study clearly indicates that BMSCs express albumin when transplanted to CCl_4-induced fibrotic liver. This study reveals that BMSCs ameliorate CCl_4-induced liver injury in mice by reducing fibrosis, expressing liver-specific genes, downregulating the expression of profibrotic genes, and upregulating antifibrotic and hepatic genes. In conclusion, the present study strengthens the concept of cellular therapy for the treatment of liver fibrosis.

ACKNOWLEDGMENTS

We thank our colleagues for the review of this manuscript. This work was supported by research grants from Higher Education Commission (HEC) of Pakistan.

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Source of Support: Research grants from Higher Education Commission (HEC) of Pakistan, **Conflict of Interest:** None declared.