Bone Marrow Mesenchymal Stem Cells Reverse Liver Damage in a Carbon Tetrachloride-induced Mouse Model of Chronic Liver Injury

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Abstract. *Background/Aim: The aim of this study was to investigate the effect of bone-marrow mesenchymal stem cells (BMSCs) on repair of liver damage in a carbon tetrachloride (CCl$_4$)-induced mouse model of chronic liver damage. Materials and Methods: Green fluorescent protein (GFP)-expressing BMSCs, isolated from GFP transgenic mice, were transplanted into mice with chronic liver damage induced by CCl$_4$. The GFP-expressing BMSCs in livers were detected by fluorescence microscopy. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured for assessment of liver function. Liver histopathology was performed to assess liver damage. mRNA and protein expression of liver-associated markers albumin (Alb) and alpha-fetoprotein (Afp) were detected to confirm the hepatic differentiation of BMSCs in the liver. Immunostaining for the expression of interleukin-10 (IL-10) and matrix metallopeptidase-9 (MMP-99), and enzyme-linked immunosorbent assay for the secretion of type III collagen and lamininin was carried out. Results: After BMSC transplantation, GFP-expressing BMSCs were detected in the peri-portal and injured areas of the CCL$_4$-injured liver. mRNA and protein expressions of Alb and Afp were significantly increased in BMSC-transplanted liver. Mice treated with BMSCs displayed reduced serum levels of ALT and AST, and CCl$_4$-induced histopathological changes in livers were repaired. BMSC transplantation increased the production of IL-10 and inhibited the expression of MMP-9, as well as the secretion of type III collagen and lamininin. Conclusion: BMSCs transplanted into mice can migrate into damaged liver, differentiate into hepatocytes and promote recovery from chemically-induced liver damage. Promotion of IL-10 and inhibition of MMP-9 by transplanted BMSCs may be involved in the anti-inflammatory and anti-fibrotic action of BMSCs.*

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Liver damage induced by chemicals or viruses often leads to liver fibrosis and sometimes leads to subsequent liver cirrhosis. Liver transplantation is the most effective therapy for cirrhosis with chronic hepatic failure, but shortage of donor organs limits the applicability of this therapy (1). Bone-marrow mesenchymal stem cells (BMSCs) have been shown to have therapeutic potential in a wide range of diseases (2-6). BMSCs are reported to have a multilineage potential and exhibit a stable phenotype *in vitro* (7). BMSC-based therapy for liver diseases is attractive because autologous BMSCs can be harvested, expanded extensively *ex vivo*, and transplanted back into the patient. *In vitro* BMSCs have the potential to differentiate into hepatocytes (8, 9). The transplantation of mesenchymal stem cells (MSCs) isolated from different sources or MSC-derived hepatocyte-like cells improved liver function of rodents with liver damage (10-12). However, the efficacy of BMSCs for liver damage is unclear (13, 14).

We investigated the feasibility and mechanism of repair of liver damage in the present study using BMSCs with a carbon tetrachloride (CCl$_4$)-induced mouse model of chronic liver

damage. The therapeutic effect was evaluated by both biochemical and histopathological analyses. In addition, the BMSCs that we used were labeled with GFP such that they could be tracked *in vivo*. Thus, we were able to visualize the homing of transplanted cells in a mouse model of liver injury.

Materials and Methods

Mice. C57BL/6–GFP female mice, aged 4-6 weeks and weighing 20-25 g, were obtained from AntiCancer Inc. (San Diego, CA, USA). All of the tissues from this transgenic line, with the exception of erythrocytes and hair, express green fluorescent protein (GFP). Non-transgenic female C57BL/6 mice, aged 4-6 weeks and weighing 20-25 g, were purchased from the Beijing Kelihua Laboratory Animal Center (Beijing, China). All mice were maintained in a HEPA-filtered environment at 24-25°C and humidity was maintained at 50-60%. All animals were fed with autoclaved laboratory rodent diet. Animal experiments were approved by the Animal Committee of Nanjing Origin Biosciences, China.

BMSC preparation. For isolation of BMSCs, C57BL/6–GFP mice were sacrificed by cervical dislocation and the limbs were removed. GFP-expressing BMSCs were flushed from the medullary cavities of the tibia and femur using a 25G needle with RPMI-1640 medium (GIBCO Life Technologies, NY, USA). The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; GIBCO), adjusted to 1×10^6 cells/ml, placed in a 25 cm^2 culture flask incubated at 37°C with 5% CO_2. Three days later, the culture solution was replaced for the first time, and cells not adhering to the flask were removed. Subsequently, the culture solution was replaced every 3 days until the cells reached 90% confluence. The medium was discarded and 1.5 ml 0.25% trypsin was added to the flask in order to harvest the cells. After fresh culture medium was added to the harvested cells, the cells were divided into two flasks and marked as passage 1 (P1). The culture medium was replaced every 3 days until the cells were grown to confluence. This procedure was repeated and the cells were marked as passage 2 (P2) and so on. GFP-expressing BMSCs were thus selected on the basis of their adherence to plastic. Sufficient BMSCs were grown in this matter in order to obtain 5×10^6 cells to inject per mouse.

Mouse model of chronic liver damage and BMSC transplantation. To generate a mouse model of chronic liver damage, CCl_4 (1.0 ml/kg body weight, 4:6 ratio of CCl_4 to olive oil) was intraperitoneally injected into C57BL/6–GFP mice twice a week for 4 weeks. Liver damage was confirmed by histological examination and liver enzyme assay four weeks before BMSC transplantation. Mice with liver damage were randomly divided into two groups of 10 mice. The BMSC-treated group received 5×10^6 BMSCs by tail vein injection. The untreated control group received saline. After BMSC transplantation, CCl_4 injections were continued at the same dose twice a week.

Immunofluorescence analysis. The mice from each group were sacrificed four weeks after BMSC transplantation. Liver tissues were collected after perfusion with 4% paraformaldehyde solution. To assess the homing of transplanted BMSCs in recipient liver, analysis with a confocal laser scanning microscope (Zeiss, LSM 510; META, Jena, Germany) was carried out to detect GFP-

expressing cells in the serial sections of the sampled liver slides. The excitation wavelength for GFP was 488 nm and fluorescence signals were recorded photographically. To determine the number of accumulated donor cells in the liver, a total of five fields of view with original magnification ×120 were randomly selected and photomicrographed. GFP-expressing cells were identified by one investigator blinded to the experimental procedures.

Histological examination. Liver histology was performed to assess liver damage 4 weeks after BMSC transplantation. The tissue was embedded in paraffin and sectioned into 4 μm slices for hematoxylin and eosin staining and Masson's trichrome staining to detect collagen in order to determine the extent of liver fibrosis, degeneration and necrosis. Histological damage was evaluated by a pathologist who was blinded to the experimental groups.

Immunohistochemistry. The liver tissue was collected at the end of the study for immunohistochemistry analysis. Liver tissues were fixed in 10% buffered formalin and paraffin-embedded. For immunohistochemistry, sections were incubated with primary antibodies against ALB, AFP, interleukin-10 (IL-10) and matrix metallopeptidase-9 (MMP-9) (all from BD Biosciences, San Diego, CA, USA) overnight at 4°C after permeabilization with a solution of 0.1% sodium citrate and 0.1%Triton X-100 and blocking with 10% rabbit serum. After washing in phosphate-buffered saline, the slices were incubated with horseradish peroxidase-labeled secondary antibody (1:200; Maixin Bio-Tech Co., Ltd, Fuzhou, China) for 30 minutes at room temperature. After color development using 3,3-diaminobenzidine for 10 minutes, the slices were counterstained in hematoxylin. The slides were viewed at ×400 magnification and positively-stained cells were recognized by the appearance of brown color development. Expression levels were quantified by the average optical density (AOD) of the positively-stained cells in five fields per sample with Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Assessment of liver function. Serum was collected 4 weeks after transplantation to analyze liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using a biochemistry autoanalyzer (Hitachi, Tokyo, Japan).

Isolation of RNA and RT-PCR. At the end of the study, the liver tissues were collected and snap-frozen in liquid nitrogen. Total RNA was isolated from liver tissue with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed using a PrimeScript RT-PCR kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions, followed by PCR amplification with specific primers. The following primers were used to amplify most of the coding region of *Alb* and *Afp*: Alb (sense, 5'-CCAAAGTCAA CAAGGAGTGC-3', and antisense, 5'-TCTTCAACAGTGGTTTA TCGCA-3'); *Afp* (sense, 5'-CACATCCA CGAGGAGTGTTGCC-3', and antisense, 5'-TAAGCCTTCAGGT TTGACGCCAT-3'); and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (sense, 5'-GGAAGGTGAAGGTCGGAGTC-3' and antisense, 5'-AATGAA GGGGTCATTCATGG-3'). Quantitative real-time PCR was performed using a 7300 real-time PCR system (Applied Biosystems, Inc., Foster City, CA, USA). PCR was carried out in a volume of 20 μl containing 10 μl 2 × qPCR Master Mix, 2 μl cDNA, 0.2 μl of each primer and 0.4 μl ROX1. Reaction conditions for amplification of target genes were one cycle of denaturing at 95°C for 5 min,
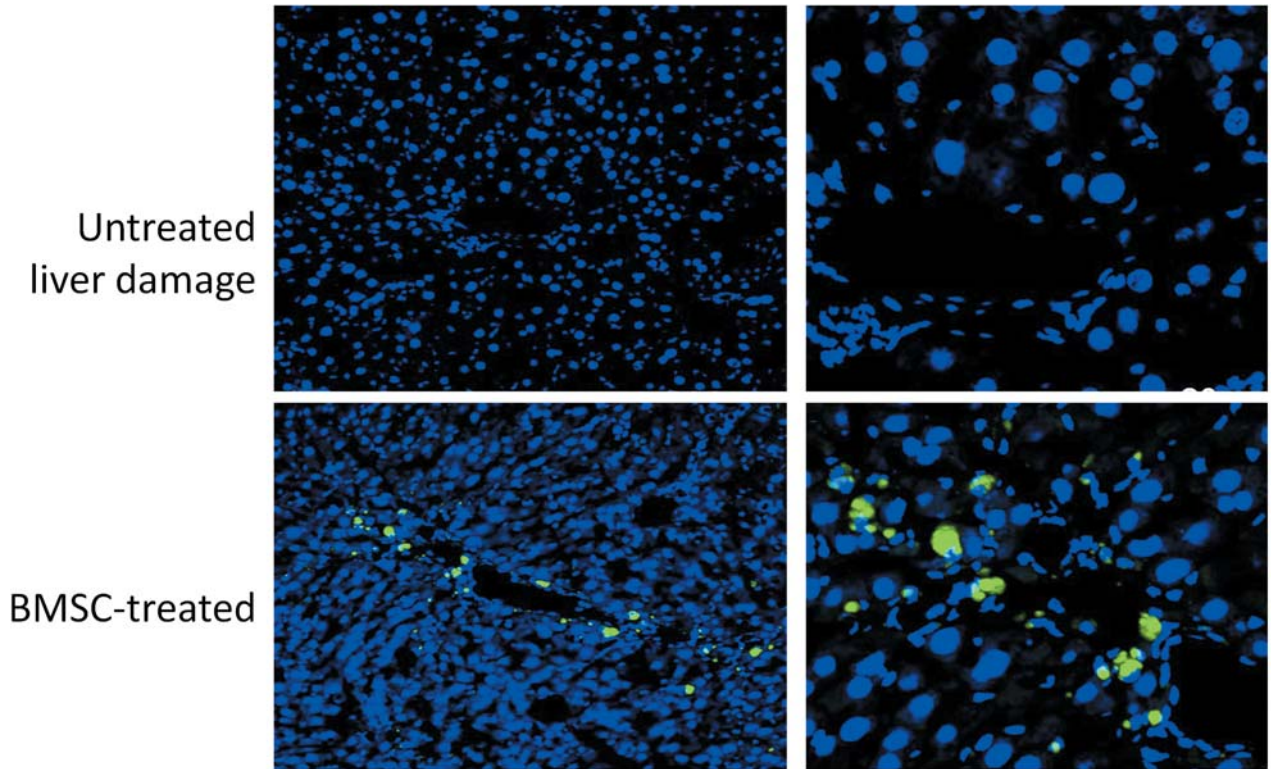
Figure 1. *Visualization of transplanted bone-marrow mesenchymal stem cells (BMSCs) in CCl$_4$-injured liver. Representative fluorescence images obtained by confocal microscopy in BMSC-treated and untreated mice at 4 weeks after BMSC transplantation. Green fluorescent protein-expressing cells indicate transplanted BMSCs. DAPI was used to visualize nuclei (blue). Right: images at ×800 magnification (bar=20 μm); left: images at ×120 magnification (bar=100 μm).*
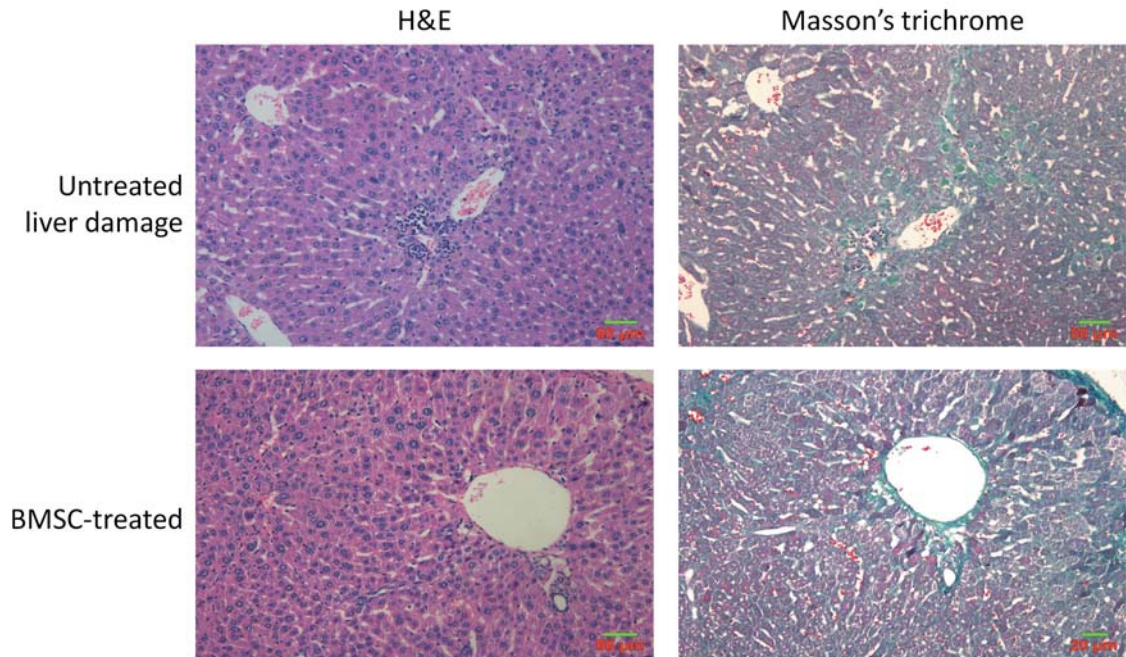

Figure 2. *Bone-marrow mesenchymal stem cell (BMSC) transplantation partly reversed liver damage induced by CCl$_4$. H&E and Masson's trichrome staining of liver sections at 4 weeks after BMSC transplantation (×100, bar=50 μm). Blue-stained regions with Masson's trichrome staining indicate collagenous fibers.*
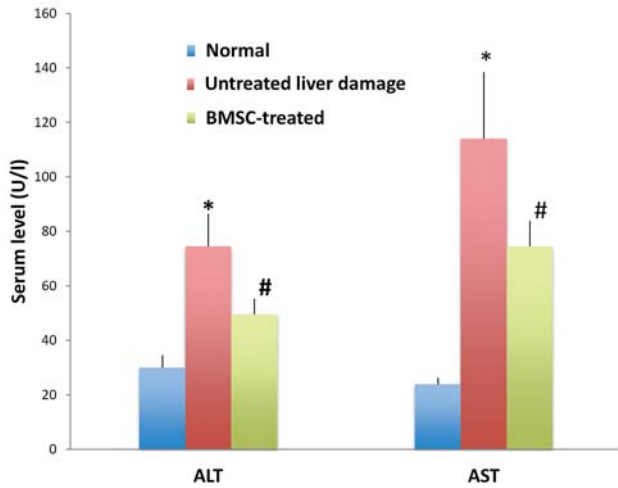
Figure 3. *Bone marrow mesenchymal stem cells (BMSCs) ameliorate CCl$_4$-induced deterioration of liver function. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity was detected using a biochemistry autoanalyzer at 4 weeks after BMSC transplantation. *p<0.01, compared to normal mice. #p<0.01 compared to mice with untreated liver damage.*
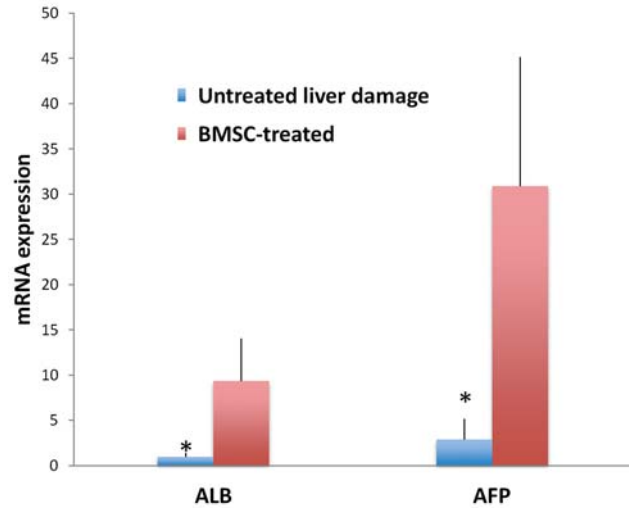

Figure 4. *Bone marrow mesenchymal stem cells (BMSCs) increase mRNA expression of albumin (Alb) and alpha-fetoprotein (Afp). Increased Alb and Afp mRNA expression in CCl$_4$-injured liver, after BMSC transplantation, was detected by real-time quantitative PCR. *p<0.01 compared to mice with untreated liver damage.*

followed by 40 cycles of 5 seconds denaturing at 95°C, 30 seconds annealing at 60°C and 40 seconds extension at 72°C. Data were analyzed with the relative standard-curve method and normalized to Gapdh expression. Relative RNA expression was calculated using the 2−ΔΔCt method. All samples were performed in triplicate.

Enzyme-linked immunosorbent assay (ELISA). The levels of type III collagen and lamininin in the liver tissue were determined in liver homogenates with an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, flat-bottom 96-well microtiter plates were coated with antibodies to type III collagen and laminin, incubated overnight at 4°C; and blocked with bovine serum albumin buffer at 37°C for 3 hours. The wells were then loaded with diluted supernatant of liver homogenates or analytic standard (100 μl/well) and incubated at 37°C for 2 hours. The plates were washed five times and incubated with biotinylated antibodies to type III collagen and laminin at 37°C for 1 hour. After being washed five times, the plates were incubated with horseradish-peroxidase-labeled avidin at 37°C for 30 minutes. After washing five times, the plates were incubated with substrate tetramethylbenzidine (100 μl/well) at 37°C for 30 minutes. The color reaction was stopped by the addition of H$_2$SO$_4$ (2 M, 50 μl/well). The optical density was measured at 450 nm using a microplate reader (ELx800uv; Bio-Tec Instruments, Houston, TX, USA). A standard curve was constructed using analytical standards, and the levels of type III collagen and laminin were calculated on the basis of the standard curve.

Statistical analysis. Statistical analysis was performed using SPSS16.0 software (Version 16.0; IBM SPSS, Chicago, IL, USA). All results are expressed as the mean ± SD. Comparisons between two groups were made with the Student's *t*-test. A value of *p*<0.05 was considered significant.

Results

Transplanted BMSCs migrate to CCl$_4$-injured liver. Confocal laser microscopy detected the transplanted GFP-BMSCs four weeks after cell transplantation by their green fluorescence. Nuclei of both hepatocytes and transplanted BMSCs were identified by blue color due to DAPI staining (Figure 1). GFP-expressing BMSCs were mainly distributed in the sinusoids of periportal areas and the foci of CCl$_4$-induced liver damage. No GFP-expressing BMSCs were found in the liver of the control mice. This suggests that the transplanted BMSCs selectively migrated to CCl$_4$-injured liver through the blood circulation and entered the sinusoid and liver parenchymal tissue.

BMSC transplantation reverses changes in histopathology of CCl$_4$-induced liver. The liver tissues were collected for histological evaluation four weeks after BMSC transplantation. As shown in Figure 2, hematoxylin and eosin and Masson's trichrome staining confirmed the presence of liver damage and hepatic fibrosis in all mice treated with CCl$_4$. Inflammatory infiltration, with mainly macrophages and lymphocytes, was seen in the areas with hepatic injury. Slight focal necrosis occurred primarily in periportal areas. Liver fibrosis was evidenced by fibrotic septa formation starting from portal areas as visualized by Masson's trichrome staining. An accumulation of collagen along the liver lobules and blood vessels was observed in CCl$_4$-treated livers. Histopathological changes were partly reversed in the
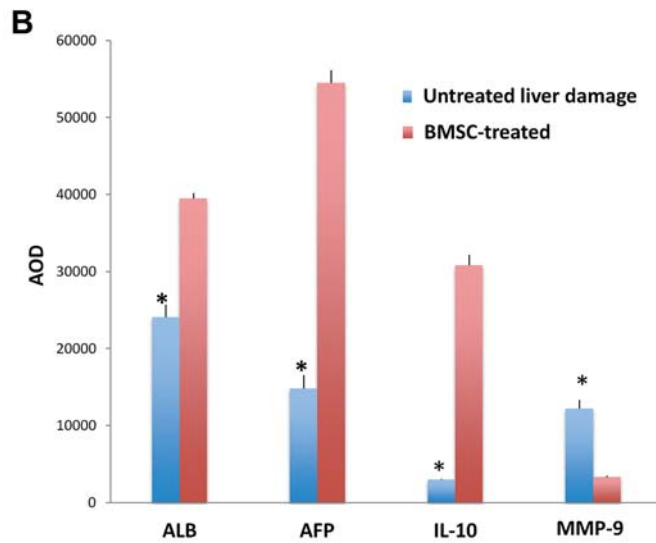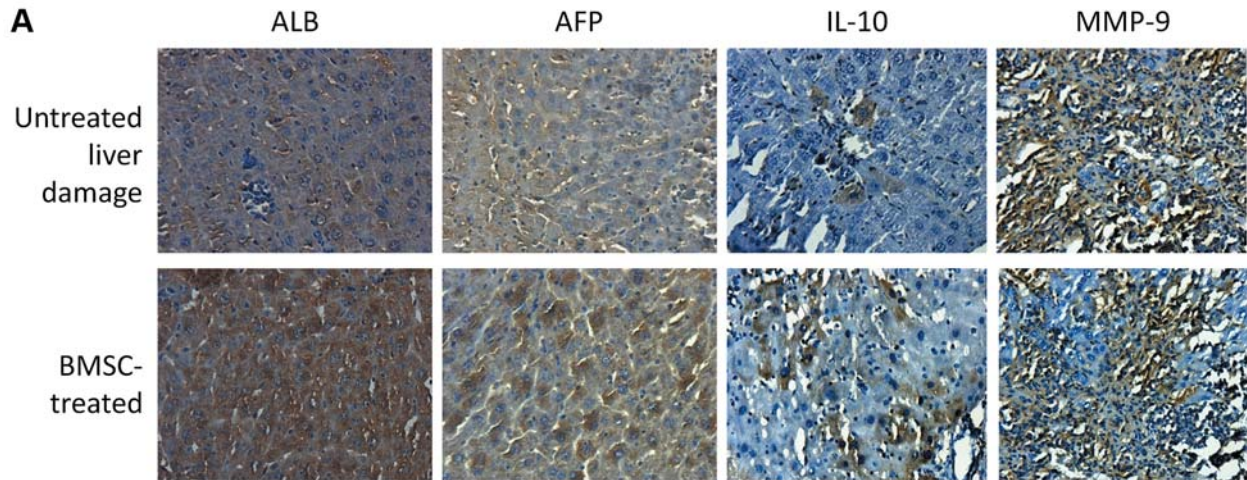
Figure 5. *Bone marrow mesenchymal stem cells (BMSCs) increase protein expression of albumin (ALB), alpha-fetoprotein (AFP), interleukin-10 (IL-10) and metallopeptidase-9 (MMP-9). A: Representative immunohistochemical staining for protein expression of ALB, AFP, IL-10 and MMP-9 in CCl_4-injured liver with or without BMSC treatment; ×400 magnification. B: Average optical density (AOD) of immunohistochemical staining of liver samples. *p<0.01 compared to mice with untreated liver damage.*

BMSC-treated mice. Reduced areas of collagen deposition were observed in the BMSC-treated mice.

BMSC transplantation ameliorates the CCl_4-induced deterioration of liver function. Liver function was monitored by measuring the serum levels of liver enzymes. As presented in Figure 3, reduced liver function was demonstrated by significantly increased levels of ALT and AST in the mice with CCl_4-induced liver damage as compared to normal mice (both *p*<0.01). BMSC treatment significantly reduced serum levels of ALT and AST as compared to untreated liver-damage control mice (both *p*<0.01). This suggests that transplantation of BMSCs results in a significant improvement of biochemical liver function indices in mice with CCl_4-induced liver injury.
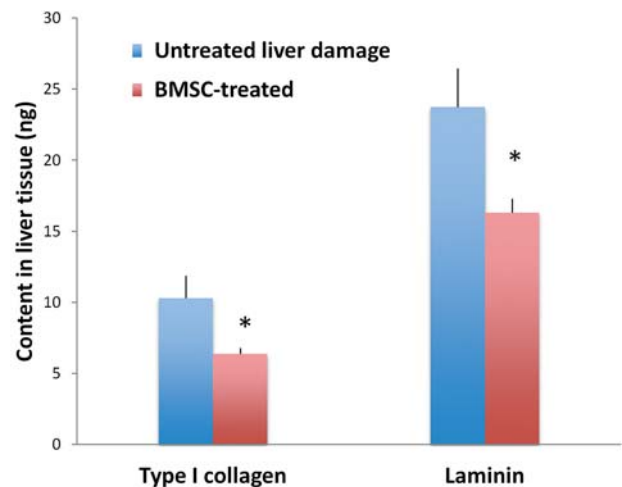

Figure 6. *Bone-marrow mesenchymal stem cells (BMSCs) reduce secretion of type III collagen and laminin. Analysis of type III collagen and laminin in CCl_4-injured liver with or without BMSC treatment by ELISA. *p<0.01 compared to mice with untreated damage.*

BMSC transplantation increases mRNA expression of Alb and Afp. The expression of liver-associated markers *Alb* and *Afp* were detected to confirm the hepatic differentiation of the BMSCs in the liver. As shown in Figure 4, four weeks following BMSC transplantation, the liver tissues with the transplanted BMSCs were found to express significantly higher mRNA levels of *Alb* and *Afp* compared to the untreated mice (both $p<0.01$). These data suggest that the transplanted BMSCs can differentiate into *Alb*- and *Afp*-secreting hepatocyte-like cells in damaged liver.

BMSC transplantation increases protein expression of ALB, AFP, IL-10 and MMP-9. The protein expression of ALB, AFP, IL-10 and MMP-9 was detected by immunohistochemical staining. As shown in Figure 5, BMSC treatment significantly induced higher protein expression of ALB and AFP than in untreated liver-damage mice (both $p<0.01$), which is consistent with the result from mRNA analysis. IL-10 is an anti-inflammatory cytokine and can suppress the progression of liver fibrosis. BMSC transplantation significantly increased the expression of IL-10 in liver compared with the untreated control ($p<0.01$). MMP-9 is known to be involved in matrix remodeling and regulation of fibrosis in the liver. Liver tissues from mice transplanted with BMSCs were found to express a significantly lower level of MMP-9 compared with those from the untreated mice ($p<0.01$). These results suggest anti-inflammatory and anti-fibrotic effects of BMSCs in the repair of damaged liver.

BMSC transplantation reduces secretion of type III collagen and laminin. The secretion of type III collagen and laminin in liver tissue was determined by ELISA. Decreased secretion of type III collagen and laminin was found in BMSC-treated mice compared with the untreated control ($p<0.01$, respectively) (Figure 6).

Discussion

Using BMSCs from GFP transgenic mice, we established a transplantation model to assess the localization and transdifferentiation of BMSCs into hepatocytes under conditions of general liver damage. In this model, chronic liver damage was induced by persistent intraperitoneal administration of CCl_4. Transplanted GFP-expressing BMSCs migrated into the periportal and injured areas of the liver. We investigated the transdifferentiation of BMSC into hepatocytes by detecting two hepatocytes markers, ALB and AFP. Immunohistochemical analysis of livers showed significantly higher ALB and AFP production in BMSC-transplanted mice, suggesting hepatocyte-like differentiation of transplanted BMSCs in the specific 'niche' of chronic liver damage.

To assess the efficacy of BMSCs in mice with CCl_4-induced liver damage, our study included biochemical analysis of liver function and histopathological examination after BMSC transplantation. Exposure to CCl_4 leads to hepatic injury, which causes alterations in permeability of the cell and mitochondrial membranes (15). As a result, cytoplasmic enzymes, including ALT and AST, are discharged into the circulation (16). Both ALT and AST reflect the degree of liver-cell injury and serve as reliable diagnostic indicators of liver damage (17). The decrease in ALT and AST levels in the serum of mice following BMSC transplantation is indicative of amelioration of liver dysfunction. Histopathological analysis of liver sections showed greater necrosis, inflammation, and fibrosis in the mice with CCl_4-induced liver injury. Masson staining also confirmed the presence of hyperplastic fibrous tissue caused by CCl_4. The mice treated with BMSCs displayed obvious alleviation of these conditions, further confirming the anti-fibrotic effects of BMSCs.

In order to investigate the mechanisms involved in the action of transplanted BMSCs on CCl_4-induced liver damage, we analyzed the expression of IL-10 and MMP-9, and secretion of type III collagen and laminin in liver. Inflammatory modulation is important in reversing hepatic fibrosis (18-20). IL-10 is an anti-inflammatory cytokine and was reported to be secreted by MSCs to modulate the activity of hepatic stellate cells (21). IL-10 actively ameliorates hepatic aggravation during fibrogenesis (22). MMP-9 plays an important role in the degradation of extracellular matrix and type IV and V collagen in fibrogenesis (23). Increased expression of MMP-9 is related to progression of liver inflammation and fibrosis, and is associated with poor prognosis. In the present study, we found that BMSCs increased the production of IL-10 and inhibited the expression of MMP-9, thus further confirming the effects of BMSCs on CCl_4-induced liver injury.

Continuous accumulation of extracellular matrix is responsible for hepatic fibrosis. As the major components of the extracellular matrix, type III collagen and laminin are important biomarkers of hepatic fibrogenesis (24, 25) and important indices for evaluating the extent of hepatic fibrosis (26, 27). Our results showed that treatment with BMSCs suppressed the levels of type III collagen and laminin in mice with CCl_4-induced liver injury, indicating that BMSCs inhibited the progression of hepatofibrosis.

In conclusion, transplanted BMSCs can migrate to damaged liver, possibly differentiate into hepatocytes and promote recovery from chemically induced liver damage. Promotion of IL-10 and reduction of MMP-9 expression by transplanted BMSCs may be involved in the anti-inflammatory and anti-fibrotic action of BMSCs.

Conflict of Interest

None of the Authors have any conflict of interest in regard to this study.

Acknowledgements

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