Original Paper

Mesenchymal Stem Cells Mitigate Cirrhosis through BMP7

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Key Words

Cirrhosis • Mesenchymal stem cells (MSCs) • CCl₄ • Transforming growth factor β 1 (TGF β 1) • Bone morphogenic protein 7 (BMP7)

Abstract

Background/Aims: Transplantation of mesenchymal stem cells (MSCs) has therapeutic effects on various diseases, while its effect on developing cirrhosis as well as the underlying mechanism remained largely unknown. **Methods:** Twenty C57BL/6 mice were randomly separated into 2 groups of ten each. One group received transplantation of MSCs, while the other group received saline as control. The mice then received intraperitoneal injection of carbon tetrachloride (CCl₄) twice per week for 8 weeks to develop cirrhosis. After another 4 weeks, the levels of cirrhosis in these mice were evaluated by liver fibrosis area, portal pressure, sodium balance and excretion. Transcripts of transforming growth factor β 1 (TGF β 1) and bone morphogenic protein 7 (BMP7) in the mouse livers were quantified by RT-qPCR. BMP7-depleted MSCs were prepared and applied in this model, and compared to MSCs. **Results:** Liver fibrosis, portal hypertension and sodium retention that were developed by CCl₄, were all significantly alleviated by MSCs transplantation, which decreased TGF β 1 levels and increased BMP7 levels in the injured liver. MSCs were found to express extremely high levels of BMP7. Knockdown of BMP7 in MSCs completely abolished the protective effect of MSCs against CCl₄-induced cirrhosis. **Conclusions:** MSCs mitigate cirrhosis through their production of BMP7 against the fibrogenic effect of TGF β 1 in the injured liver.

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Introduction

Mesenchymal stem cells (MSCs) have been initially identified in the bone marrow, and they are capable of extensively expanding in culture and differentiating into osteoblasts, chondrocytes, and adipocytes in response to corresponding stimulants. Moreover, MSCs have been shown to possess a particular property of immunosuppression in that it may alter immunity and inflammation during disease progresses [1]. Recent studies further demonstrate a potential role of MSCs in promoting hematopoietic engraftment and tissue repair [1-3]. When intravenously delivered, retention of MSCs mainly occurs in the lung [4, 5]. Therefore, orthotopic transplantation is needed to allow MSCs to properly function in organs other than the lung [6].

Hepatic fibrosis is overly exuberant wound healing in which excessive connective tissue accumulates in the liver [7-10]. The extracellular matrix is overproduced, degraded deficiently, or combined. The common trigger for hepatic fibrosis is injury-induced chronic inflammation, leading to portal hypertension due to distortion of blood flow through the liver by the scarring, or cirrhosis that results in disruption of normal hepatic architecture and liver dysfunction [7-10].

The current approaches to study the molecular mechanisms underlying the pathogenesis of cirrhosis often rely on the use of animal model [11-16]. So far, the most commonly used methods to induce chronic liver damage in mice are intragastric, intraperitoneal or subcutaneous administration of carbon tetrachloride (CCl_4) or other hepatotoxins, among which intraperitoneal injection of CCl_4 has advantages to be low toxic to mice (least lethal) and reproducible induction of cirrhosis, although the levels of cirrhosis by this method may be not as pronounced as some other methods [11-16].

The cellular regulation of the development of hepatic fibrosis includes signaling molecules and receptors responsible for inflammatory cell recruitment, hepatocyte death and survival factors, and modulators of epithelial-mesenchymal transition (EMT), which plays an important role in the progression of fibrosis in the liver [17]. Major regulators of EMT in the liver are two multifunctional growth factors from transforming growth factor β ($\text{TGF}\beta$) superfamily [18-20], bone morphogenic protein 7 (BMP7) and $\text{TGF}\beta 1$. While $\text{TGF}\beta 1$ is a well-established fibrotic inducer to promote transdifferentiation of hepatic stellate cells into myofibroblasts, BMP7 reverses EMT by directly counteracting $\text{TGF}\beta 1$ -induced Smad-dependent cell signaling [21-25]. Such antagonism coordinates the repair of the injured liver [26-30].

However, although transplantation of MSCs has therapeutic effects on various diseases, its effect on cirrhosis as well as the underlying mechanisms remains largely unknown. Here we examined the effects of MSCs on CCl_4 -induced cirrhosis in mice, and specifically evaluated the potential of BMP7 produced by MSCs.

Materials and Methods

MSCs isolation, culturing, differentiation and transduction

The MSCs were isolated and grown in culture as has been described previously [3, 6]. Briefly, plugs of marrow from five 12-week-old male C57BL/6 mice (about 30g body weight) were dispersed in Dulbecco's Modification of Eagle's Medium (DMEM, Life technologies, San Jose, CA, USA) and then centrifuged at 900g for 5 min. The pellets were re-suspended and plated at 10^5 cells/cm² in DMEM containing 10% FBS. After 10 passages' selection of attached cells, the cells were sorted for Stro-1 (Becton-Dickinson Biosciences, San Jose, CA, USA) by flow cytometry to get rid of contaminating cells. A positive clone was selected after subjection to chondrogenetic, osteogenic, and adipogenic differentiation assays to confirm phenotype. For chondrogenetic induction, 2.5×10^5 MSC were induced with 5ml chondrogenetic induction medium containing 10 μg $\text{TGF}\beta 1$ (R&D System, Los Angeles, CA, USA), 50 μg insulin growth factor 1 (R&D System), and 2mg/mL dexamethasone (DMSO, Sigma-Aldrich, St. Louis, MO, USA) followed by centrifugation at 500g for

5 min. The cell pellets were maintained in the chondrogenetic induction medium for 14 days and subjected to Alcian blue staining. For osteogenic induction, cells were digested and seeded onto a 24-well plate at a density of 10^4 cells/well, and then maintained in osteogenic induction medium containing 10 nM Vitamin D3 (Sigma-Aldrich) and 10 mM β -phosphoglycerol and 0.1 μ M DMSO for 14 days and were subjected to Von kossa staining. For adipogenic induction, cells were digested and seeded onto a 24-well plate at a density of 10^4 cells/well, and then maintained in the adipogenic induction medium containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 200 μ M indomethacin, 10 μ M insulin and 1 μ M DMSO for 14 days and subjected to Oil red O staining. For knock-down of BMP7 in MSCs, recombinant lentiviruses expressing either short hairpin small interfering RNA for BMP7, or scrambled sequence, were used to transduce MSCs at MOI 100 with nearly 100% infection efficiency, resulting in MSCs-shBMP7 and MSCs-scr, respectively.

Animal manipulations

All animal procedures were conducted according to the guidelines for the care and use of laboratory animals approved by 302 Hospital of PLA. C57BL/6 mice (Charles River Laboratories, China) of 10 weeks of age were given free access to tap water and pelleted mouse diet. During cirrhosis induction, the mice were provided with 0.3 g/L phenobarbital in drinking water to enhance CCl_4 hepatotoxicity. For MSCs transplantation, MSCs (10^7) were directly injected into the parenchyma of the liver at six spots.

Induction of cirrhosis in mice

Cirrhosis was induced by CCl_4 intraperitoneal administration. Briefly, CCl_4 solution of 50% (v/v) in paraffin oil (Sigma-Aldrich, St. Louis, MO, USA) was administered at a dose of 2 ml per kg body weight of the mice, by intraperitoneal injections twice a week for 8 weeks.

Evaluation of liver fibrosis

Liver samples were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and stained with the Sirius red staining technique. Fibrotic areas were counted on 200 random selected fields corresponding to approximately 6 mm², using an unbiased counting frame, as has been described previously [14]. The percentage of fibrotic area was expressed as number of fibrotic fields divided by total fields, and then multiplied by 100.

Sodium balance

The urine sodium concentration (UNa) was assayed by flame photometry (Roika 2000, Roika, UK), and renal sodium excretion (UNaV) was calculated with the following formula: $\text{UNaV} = \text{UV} \times \text{UNa}$, where UV is urine volume. The intake of sodium was assessed by measuring the amounts of food and water consumed. Sodium balance was calculated as (Na⁺ provided by food and water) - UNaV.

Portal pressure

After evaluation of sodium metabolism, under anesthesia, a PE-50 polyvinyl catheter was placed in the cecal vein. The other end of the catheter was connected to a highly sensitive transducer (ADInstruments Shanghai Trading Co. Shanghai, China) to assess portal pressure.

Quantitative real-time PCR (RT-qPCR)

RNA was extracted from the liver tissue or the cultured MSCs with Trizol (Invitrogen, St Louis, MO, USA), and then used for cDNA synthesis. RT-qPCR was performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Values of genes were normalized against α -tubulin and then compared to the controls.

Statistical analysis

Statistical analyses were performed with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). All data were statistically analyzed using one-way ANOVA with a Bonferoni correction. χ^2 test with Fisher's correction was used for comparison of survival in each experimental group. All values are depicted as mean $\pm$ standard deviation from 5 individuals and are considered significant if $p < 0.05$.

Fig. 1. MSCs culture and differentiation assays. (A-C) MSCs differentiation assay. (A) Alcian blue staining to evaluate chondrogenetic induction. (B) Von kossa staining to evaluate osteogenic induction. (C) Oil red O staining to evaluate adipogenic induction. Scale bars are 30µm.

Fig. 2. Schematic of the model. Mice were first transplanted with MSCs of 10^7 , or control saline, directly into the liver, and then subjected to i.p. injection of CCl_4 twice per week for 8 weeks. During this period, 0.3 g/L phenobarbital was provided in drinking water to enhance CCl_4 hepatotoxicity. Afterwards, the mice were kept for another 4 weeks, and then analyzed.

Results

Isolation, culturing and differentiation of primary mouse MSCs

Primary mouse MSCs were isolated from male C57BL/6 mice and expanded in culture. To confirm MSC phenotype, differentiation assays were performed, including Alcian blue staining to evaluate chondrogenetic induction (Fig. 1A), Von kossa staining to evaluate osteogenic induction (Fig. 1B), and Oil red O staining to evaluate adipogenic induction (Fig. 1C).

MSCs transplantation significantly alleviated the features of cirrhosis induced by CCl_4

In order to evaluate the effect of transplantation of MSCs on cirrhosis, we used a well-established CCl_4 -induced cirrhosis animal model. Mice were first transplanted with 10^7 MSCs, or control saline, directly into the liver, and then subjected to i.p. injection of CCl_4 twice per week for 8 weeks. During this period, 0.3 g/L phenobarbital was provided in drinking water to enhance CCl_4 hepatotoxicity. Afterwards, the mice were kept for another 4 weeks, and then analyzed (Fig. 2). Mice mortality at sacrifice was very limited in both groups (1 from 10 each), with or without MSCs transplantation.

At sacrifice, all mice presented features of cirrhosis. The fibrotic area at sacrifice was evaluated after Sirius red staining, showing that MSCs transplantation significantly decreased the percentage of the fibrotic area (Fig. 3A). Portal hypertension and sodium metabolism were also assessed, showing that MSCs transplantation significantly decreased the portal pressure (Fig. 3B), significantly improved sodium balance (Fig. 3C), probably through an increased sodium excretion (Fig. 3D). These data suggest that MSCs transplantation significantly inhibited the development of cirrhosis induced by CCl_4 .

MSCs transplantation increased BMP7 and decreased TGFβ1 in the injured liver

TGFβ1 is a well-known fibrosis promoter, while BMP7 has a potential role in antagonizing the effect of TGFβ1 in fibrosis. Thus, we examined whether MSCs may affect the balance

Fig. 3. MSCs transplantation significantly alleviated the features of cirrhosis induced by CCl_4 . (A) The fibrotic area at sacrifice was evaluated after Sirius red staining, shown by the percentage of the fibrotic area. (B) Portal pressure. (C) Sodium balance. (D) Sodium excretion. *: $p < 0.05$. $n = 9$. Statistics: one-way ANOVA with a Bonferroni correction.

Fig. 4. MSCs transplantation increased BMP7 and decreased TGFβ1 in the injured liver. (A-B) Mouse livers at sacrifice (with/without MSCs) were examined for TGFβ1 (A) and BMP7 (B) transcripts, compared to the cultured MSCs, or MSC-shBMP7. (C) Schematic: Mice were first transplanted with MSCs-shBMP7 or MSCs-scr of 10^7 each, directly into the liver, and then subjected to i.p. injection of CCl_4 twice per week for 8 weeks. During this period, 0.3 g/L phenobarbital was provided in drinking water to enhance CCl_4 hepatotoxicity. Afterwards, the mice were kept for another 4 weeks, and then analyzed. *: $p < 0.05$. $n = 9$. Statistics: one-way ANOVA with a Bonferroni correction.

of TGFβ1 and BMP7 in the injured liver to inhibit development of fibrosis. Mouse livers at sacrifice were thus analyzed for TGFβ1 and BMP7 transcripts, compared to the cultured MSCs. We found significantly decreased TGFβ1 (Fig. 4A) and significantly increased BMP7 (Fig. 4B) transcripts in the livers from the mice that had received MSCs transplantation,

Fig. 5. Knock-down of BMP7 in MSCs abolished their anti-cirrhosis effect. (A) The fibrotic area at sacrifice was evaluated after Sirius red staining, shown by the percentage of the fibrotic area. (B) Portal pressure. (C) Sodium balance. (D) Sodium excretion. *: $p < 0.05$. $n = 9$. Statistics: one-way ANOVA with a Bonferroni correction.

which supports our hypothesis. Moreover, it appeared that MSCs are a major source of BMP7 in the injured liver (Fig. 4B).

Knock-down of BMP7 in MSCs abolished its anti-cirrhosis effect

In order to find out whether BMP7 produced by MSCs is responsible for the inhibitory effect of MSCs transplantation on the development of cirrhosis, we inhibited the expression of BMP7 in MSCs using shRNA for BMP7 (MSCs-shBMP7). MSCs that were transduced with scrambled sequence (MSCs-scr) were used as controls, showing no difference with MSCs (not shown). The knock-down of BMP7 in MSCs was confirmed by examining BMP7 levels (Fig. 4B).

Then we transplanted these MSCs-shBMP7 or MSCs-scr into mice and induced cirrhosis with CCl₄ (Fig. 4C). We found that knock-down of BMP7 in MSCs completely abolished its effects on fibrosis (Fig. 5A), portal pressure (Fig. 5B) and sodium metabolism (Fig. 5C-D), suggesting that the inhibitory effect of MSCs transplantation on the development of cirrhosis results from its production of BMP7, which may contradict the fibrotic effect of TGF β 1.

Discussion

CCl₄-induced cirrhosis is a traditional cirrhosis model, which has been most commonly applied in the previous studies on chronic liver diseases [7-10]. Compared with genetically modified mouse models, CCl₄-induced cirrhosis model has many merits, and best resembles human liver fibrotic diseases [11-16]. Among different ways of CCl₄ administration including inhalation, subcutaneous injection and intraperitoneal injection, the latter has several advantages, e.g. fast development of cirrhosis, less toxicity and low lethality [11-16]. Although

CCl₄ by i.p may not be able to achieve highly severe cirrhosis and pronounced ascites, these parameters are not required for our study [11-16].

MSCs have been shown capable of regulating immunity and inflammation during disease progresses, and of promoting tissue repair [1-3]. In the current study, we injected MSCs directly into the parenchyma of the liver, since intravenous delivery of MSCs may suffer from pulmonary retention, resulting in poor cell perfusion into the liver [4-6].

We found that i.p injection of CCl₄ successfully induced cirrhosis in all the mice, with typical histological changes of fibrosis, increased portal hypertension and sodium retention. However, MSCs substantially inhibited all these hallmarks for cirrhosis, suggesting that MSCs inhibits development of fibrosis. Since TGFβ1 is the most important activator for tissue fibrosis, and its fibrotic effect is often contradicted by BMP7 [18-20], we were prompted to examine whether MSCs may alter TGFβ1 and BMP7 levels in the injured liver. Our data confirmed our hypothesis, by showing a significant reverse of TGFβ1-to-BMP7 ratio in the liver by MSCs transplantation, which may potentially result from the abundance of BMP7 produced by MSCs. We used transcripts of TGFβ1 and BMP7, rather than proteins for quantification, since there had been no reports about the post-translational controls of these two proteins.

In order to prove that BMP7 produced by MSCs is responsible for the inhibitory effect of MSCs transplantation on the development of cirrhosis, we inhibited the expression of BMP7 in MSCs by nearly 90%. Interestingly, knock-down of BMP7 in MSCs completely abolished their effects on all the hallmarks of cirrhosis, which highlights a pivotal role of MSCs-derived BMP7 in the protection from cirrhosis.

In summary, our study not only demonstrates an inhibitory effect of MSCs transplantation on the development of cirrhosis, but also suggests that this protective effect results from the production of BMP7 by MSCs.

Finally, it should be taken into account that the mouse strain may affect the results in this study, and particularly may affect the responses of the mice to the CCl₄ toxicity to influence the progression to and the level of fibrosis and cirrhosis. Future studies may be performed to evaluate this model in different strains, and in immunocompromised mice in responsive to transplantation of human MSCs.

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Disclosure Statement

The authors have declared that no competing interests exist.

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