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Synergistic effects of simvastatin and bone marrow-derived mesenchymal stem cells on hepatic fibrosis

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ABSTRACT

The beneficial effects of simvastatin on fibrosis in various organs have been reported. In addition, bone marrow (BM)-derived mesenchymal stem cells (MSCs) have been suggested as an effective therapy for hepatic fibrosis and cirrhosis. Recent evidence suggests that pharmacological treatment devoted to regulating stem cell function is a potential new therapeutic strategy that is drawing nearer to clinical practice. The aim of this study was to determine whether the combination treatment of simvastatin plus MSCs (Sim-MSCs) could have a synergistic effect on hepatic fibrosis in a thioacetamide (TAA)-induced cirrhotic rat model and hepatic stellate cells (HSCs). Cirrhotic livers from rats treated with Sim-MSCs exhibited histological improvement compared to those treated with simvastatin alone. Sim-MSCs combination treatment decreased hepatic collagen distribution, lowered the hydroxyproline content, and rescued liver function impairment in rats with TAA-induced cirrhosis. These protective effects were more potent with Sim-MSCs than with simvastatin alone. The upregulation of collagen-1, α-smooth muscle actin (α-SMA), transforming growth factor (TGF)-β1, and phospho-Smad3 in cirrhotic livers was prevented by the administration of Sim-MSCs. Intriguingly, Sim-MSCs inhibited both TGF-β/Smad3 signaling and α-SMA in HSCs. The Sim-MSCs combination treatment exerted strong protective effects against hepatic fibrosis by suppressing TGF-β/Smad signaling. Simvastatin could act synergistically with MSCs as an efficient therapeutic approach for intractable cirrhosis.

1. Introduction

Cirrhosis is the late stage of progressive hepatic fibrosis, which is characterized by distortion of the hepatic construction and the composition of regenerative nodules, angiogenesis, and shunts [1–3]. Since hepatic fibrosis is a common development in a variety of chronic liver diseases, its therapy is of great significance. Liver transplantation has been the only treatment for patients with advanced liver diseases. However, liver transplantation has critical limitations that have not yet been overcome.

Transforming growth factor (TGF)-β1 is a key mediator of fibrogenesis, and the TGF-β1 signaling pathway contributes to liver fibrosis progression. More importantly, TGF-β1 mediates its biological functions via the canonical Smad pathway by activating the transmembrane receptors that stimulate the cytoplasmic Smad proteins, which in turn activate collagen transcription [4]. Therefore, the TGF-β1 activated Smad3 signaling pathway is critical for the formation of hepatic fibrosis, and TGF-β signaling pathways are potential therapeutic targets for liver fibrosis.

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Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, have garnered attention for their pleiotropic effects. It has been reported that statins have beneficial effects independent of their ability to reduce cholesterol, including enhancement of endothelial dysfunction, increased nitric oxide bioavailability, immunomodulatory properties, antioxidant effects, and anti-inflammatory activity. Furthermore, statins attenuate TGF-β1 signaling by inhibiting the Rho/ROCK pathway, which results in reduced expression of growth factors such as CTGF, reduced collagen transcription, and less extensive collagen contraction [5]. Therefore, they can be potent therapeutic agents for fibrotic disease. However, a previous study reported an insufficient antifibrotic effect in a patient with chronic liver disease. Hence, the clinical suitability of statins is still being critically evaluated.

Stem cell transplantation has been proposed as an alternative therapy for liver disease. Mesenchymal stem cells (MSCs) have many practical advantages in regenerative medicine, including their low immunogenicity, multipotent differentiation capacity, and minimal ethical problems [6,7]. In addition, we have previously demonstrated that bone marrow (BM)-derived MSC therapy improves hepatic fibrosis *in vitro*, *in vivo*, and in clinical studies [8–10]. However, major limitations to the efficacy of cell therapy are the low survival rates and short duration of survival of the transplanted cells. A previous study showed that MSC numbers gradually reduced and disappeared at 2 weeks after injection in fibrotic rat livers [9]. Hence, the functional improvement of stem cell therapy may require an important strategic advancement in regenerative medicine. We recently used MSCs in combination with decorin-expressing adenovirus to treat hepatic fibrosis and demonstrated that this combination therapy significantly prevented hepatic fibrosis to a greater extent than either therapy alone while also augmenting MSC viability and tissue repair [11]. Furthermore, recent reports have shown that statins could modulate the biological characteristics and functions of various stem cells and thus could be an effective method to facilitate stem cell therapy [12]. Hence, we hypothesized that a combination treatment consisting of MSCs with simvastatin (Sim-MSCs) could be used as a synergistic therapy and that this system would exhibit improved efficacy and safety compared with simvastatin therapy alone. In this study, we explored the synergistic effect of a combination treatment consisting of Sim-MSCs on hepatic fibrosis in a rat model of thioacetamide (TAA)-induced cirrhosis and hepatic stellate cells (HSCs). Moreover, we investigated the underlying mechanisms for this process.

2. Materials and methods

2.1. Experimental animals

Male Sprague-Dawley (SD) rats (7 weeks old) were purchased from Orient Bio Inc. (Seongnam, Korea) and maintained at room temperature (RT) (25 °C) with a 12/12-h light/dark cycle. Hepatic fibrosis was induced in SD rats by intraperitoneal injection of TAA (Sigma-Aldrich, St. Louis, MO, USA; 300 mg/kg body weight) twice a week for 12 weeks. All animal experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee of Yonsei University Wonju College of Medicine.

2.2. Collection and isolation of human BM-derived MSCS

Human MSCs were obtained from healthy persons who voluntarily donated their BM stem cells as previously described [11]. All protocols and procedures involving human subjects were approved by the Institutional Review Board of Yonsei University Wonju Severance Hospital (CR109021) and were conducted according to the principles of the Declaration of Helsinki. All participants provided written informed consent before study participation.

2.3. BM-MSC immunophenotyping and differentiation assays

The immunophenotypes of the MSCs (identified with the cell surface markers cluster of differentiation (CD)14, CD34, CD45, CD73, and CD105) were analyzed on the day of injection, and their differentiation potentials were also identified (osteogenic and adipogenic; Fig. 1) as previously described [11,13,14].

2.4. Administration of simvastatin and MSCs in rat model of TAA-induced cirrhosis

Animals were randomly allocated to four groups (each group, $n = 10$) as follows: group I (G1, sham group); group II (G2, untreated cirrhotic group), which received the TAA injections; group III (G3, simvastatin-treated group), which received both the TAA injections and the simvastatin treatment; and group IV (G4, simvastatin plus MSCs-treated group), which received both the TAA injections and the Sim-MSCs treatment.

G3 and G4 were given simvastatin (Sigma-Aldrich) at $10\,mg\,kg^{-1}\cdot day^{-1}$ via drinking water for 5 weeks. Daily water consumption was monitored to adjust the dose of simvastatin delivered daily.

Rats were anesthetized by intramuscular injection of a mixture of Zoletil (Virbac Laboratories, Carros, France) and Rompun (Bayer Korea, Seoul, Korea). Using aseptic techniques, a 1-cm incision was made caudal to the costal arch on the right flank to expose the right lobe of the liver. With a syringe, 1×10^6 MSCs were injected directly into the right lobe of the liver at 6 and 8 weeks during the 12-week course of TAA administration (Fig. 1D). After 12 weeks, blood samples were taken, and the rats were sacrificed. Liver tissue specimens were collected, fixed, immediately frozen, and stored at −80 °C for analysis.

2.5. Histomorphological and immunohistochemical analysis

Thick sections (5 μm) of paraffin-embedded liver tissue were prepared and stained with hematoxylin and eosin (H&E), Picrosirius red, and Masson's trichrome (MTC). The extent of fibrosis was evaluated by using the Laennec fibrosis scoring system (Supplementary Table 1). The Laennec fibrosis scoring system was used because it incorporates three subclasses of cirrhosis, thereby enabling a more detailed estimation of the effects of the intervention on fibrosis [15]. To further assess the effects of each treatment on hepatic fibrosis, the fibrotic area in each liver specimen was quantified as a percentage of the total MTC-stained area. Picrosirius red staining was performed to quantify the total amount of collagen. Thick sections of paraffin-embedded liver tissue were deparaffinized, rehydrated with distilled water, and stained with a Picrosirius red staining kit (Polysciences, Warrington, PA, USA) according to the manufacturer's instructions [11,16]. For immunofluorescence staining, frozen liver sections were fixed in cold acetone, and nonspecific binding sites were blocked by incubation in 10% fetal bovine serum (FBS) for 2 h at RT as previously described [11].

2.6. Measurement of hepatic hydroxyproline content

Hepatic hydroxyproline content was spectrophotometrically measured using prepared Ehrlich's solution (dimethylaminobenzaldehyde with perchloric acid and isopropanol) as previously described [11].

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