ORIGINAL ARTICLE

Conditioned Medium from Tonsil-Derived Mesenchymal Stem Cells Relieves CCl₄-Induced Liver Fibrosis in Mice

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Abstract

BACKGROUND: The liver is an organ with remarkable regenerative capacity; however, once chronic fibrosis occurs, liver failure follows, with high mortality and morbidity rates. Continuous exposure to proinflammatory stimuli exaggerates the pathological process of liver failure; therefore, immune modulation is a potential strategy to treat liver fibrosis. Mesenchymal stem cells (MSCs) with tissue regenerative and immunomodulatory potential may support the development of therapeutics for liver fibrosis.

METHODS: Here, we induced hepatic injury in mice by injecting carbon tetrachloride (CCl₄) and investigated the therapeutic potential of conditioned medium from tonsil-derived MSCs (T-MSC CM). In parallel, we used recombinant human IL-1Ra, which, as we have previously shown, is secreted exclusively from T-MSCs and resolves the fibrogenic activation of myoblasts. Hepatic inflammation and fibrosis were determined by histological analyses using H&E and Picro-Sirius Red staining.

RESULTS: The results demonstrated that T-MSC CM treatment significantly reduced inflammation as well as fibrosis in the CCl₄-injured mouse liver. IL-1Ra injection showed effects similar to T-MSC CM treatment, suggesting that T-MSC CM may exert anti-inflammatory and anti-fibrotic effects via the endogenous production of IL-1Ra. The expression of genes involved in fibrosis was evaluated, and the results showed significant induction of alpha-1 type I collagen, transforming growth factor beta, and tissue inhibitor of metalloproteases 1 upon CCl₄ injection, whereas treatment with T-MSC CM or IL-1Ra downregulated their expression.

CONCLUSIONS: Taken together, these data support the therapeutic potential of T-MSC CM and/or IL-1Ra for the alleviation of liver fibrosis, as well as in treating diseases involving organ fibrosis.

Keywords Tonsil-derived mesenchymal stem cells · Liver fibrosis · Interleukin-1 receptor antagonist

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1 Introduction

Liver injury can be induced by a variety of causes such as viral infection, alcohol consumption, drug abuse, or obesity. Hepatocellular injury is usually followed by inflammation, and immune cells, as well as hepatic stellate cells (HSCs), are activated to stimulate cytokine production and extracellular matrix (ECM) protein synthesis. Continuous ECM deposition can aggravate liver fibrosis, and ECM degradation does not occur properly. Once irreversible liver failure happens, the only definitive treatment option is organ transplantation, but the shortage in donor organs is a hurdle in the bid to increase survival rates of patients with liver cirrhosis. Therefore, the development of effective anti-fibrotic and immune-modulating drugs is much needed.

Mesenchymal stem cells (MSCs) are an attractive source from which to develop therapeutics, because of their immunomodulatory and tissue regenerative properties. Among the various tissue sources of MSCs, human palatine tonsils possess advantages over bone marrow (BM) or adipose tissue (AD). We have reported that tonsil-derived MSCs (T-MSCs) proliferate 3–4 times faster than BM- or AD-MSCs and secrete a more diverse range of proteins that function in immune modulation [1, 2]. In particular, T-MSCs produce immunomodulatory effects by secreting IL-1 receptor antagonist (IL-1Ra), programmed death ligand-1 (PD-L1), and osteoprotegerin at much higher levels than other MSCs [3–5]. Previously, we demonstrated that T-MSCs show tissue regenerative effects in carbon tetrachloride (CCl₄)- or concanavalin A-induced liver injury mouse models [6]. Resolution of hepatic inflammation and fibrosis was observed in transplanting T-MSCs, which can differentiate into hepatocytes. Another possible mechanism of action might involve paracrine factors that can regulate inflammation and fibrosis. A recent study using RNA sequencing analysis revealed that T-MSCs produce significantly higher levels of the proteins involved in ECM organization [2]; thus, their effects in fibrosis should be evaluated.

Activated inflammatory cells and HSCs secrete and respond to cytokines such as transforming growth factor (TGF)- β and interleukin-1 (IL-1). TGF- β produces pro-fibrogenic effects by upregulating the production of ECM component proteins while downregulating matrix metalloproteinases (MMPs) that are involved in ECM remodeling [7]. IL-1 expression is induced at early stages of liver inflammation and initiates liver fibrosis by triggering HSCs [8]. Involvement of the IL-1 signaling pathway in the progression of liver fibrosis has been demonstrated in several studies. Mice lacking the IL-1 receptor have been shown to be protected from liver fibrosis [9, 10]. Although

the mechanisms of the IL-1 signaling pathway that regulates liver fibrosis have not been completely elucidated yet, its involvement in pathologic fibrosis has been well studied in rheumatoid arthritis, periodontitis, and pulmonary fibrosis [11–13]. In the previous study, we have shown that T-MSCs endogenously produce IL-1Ra at levels much higher than those in BM- or AD-derived MSCs [3]. We demonstrated resolution of fibrogenic activation in skeletal muscle cells under a pathophysiological condition induced by palmitic acid treatment.

In this study, we aimed to identify the effectiveness of T-MSC CM or recombinant human IL-1Ra (rhIL-1Ra) in treating liver fibrosis initiated by chronic CCl₄ injection. To address this, we examined histological changes in the mouse liver and evaluated inflammation and fibrosis following treatment. The expression levels of genes involved in inflammation and fibrosis were analyzed in parallel.

2 Materials and methods

2.1 Cell culture and CM preparation

T-MSCs previously isolated from human tonsils (IRB approval by Ewha Womans University Mok-Dong Hospital, Seoul, Korea; no. ECT11-53-02) were cultured in DMEM medium (Welgene, Gyeongsan, Korea) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μ g/mL streptomycin (Welgene), and incubated at 37 °C in an atmosphere of humidified 5% CO₂. At 80% confluence, the cells were washed twice with PBS and incubated with serum-free DMEM for an additional 48 h. The CM was collected and passed through a 0.2- μ m syringe filter to remove cell debris, and then concentrated 20-fold using centrifugal filter units with a 3-kDa cut-off (EMD Millipore, Darmstadt, Germany), by centrifuging at 4500 rpm at 4 °C for 1 h.

2.2 Flow cytometry

For flow cytometry analysis of MSC surface markers, T-MSCs were collected by trypsin incubation for 2 min, and cells were spun down by centrifuging at 1300 rpm for 5 min at room temperature. Cells were resuspended in PBS-based buffer containing 0.5% FBS and 0.1% (v/v) sodium azide. The following antibodies were added to the cells followed by incubation for 30 min on ice: Alexa Fluor 488 anti-human CD34 (561, mouse IgG2a; Biolegend, San Diego, CA, USA), peridinin chlorophyll protein (PerCP)-labeled anti-human CD45 (2D1, mouse IgG1; Biolegend, San Diego, CA, USA), allophycocyanin (APC)-labeled anti-human CD73 (AD2, mouse IgG1; Biolegend, San Diego, CA, USA), and phycoerythrin (PE)-labeled anti-

human CD90 (5E10, mouse IgG1; BD Biosciences, San Jose, CA, USA). Cells were washed once with buffer; subsequently, marker expression was measured on a NovoCyt flow cytometer and analyzed using NovoExpress software (ACEA Biosciences, San Diego, CA, USA).

2.3 Mesodermal differentiation of T-MSCs

To induce the differentiation of T-MSCs to adipocytes or osteoblasts, confluent T-MSCs were incubated using the StemPro adipogenesis or osteogenesis differentiation kit for 3 weeks, respectively (Thermo Fisher Scientific, Waltham, MA, USA). To determine adipocyte differentiation, cells were fixed in 4% PFA and stained with Oil Red O staining solution for 10 min at room temperature. Osteoblast differentiation was examined by fixing cells with 60% isopropanol and then staining with Alizarin Red S solution for 3 min. For the induction of chondrocyte differentiation, 2×10^6 cells were pelleted by centrifugation at 1300 rpm for 5 min at room temperature and incubated with the StemPro chondrogenesis differentiation kit (Thermo Fisher Scientific, Waltham, MA, USA) for 3 weeks. The chondrocyte pellet was fixed using 4% paraformaldehyde (PFA), and paraffin-embedded sections (5 μ m thickness) were prepared. Deparaffinized slides were stained in Alcian blue solution for 30 min, then counterstaining was performed in nuclear fast red solution for 5 min.

2.4 Animals

A total of 12 male 4-week-old C57BL/6 mice were purchased (Orient Bio, Sungnam, Korea) and maintained under conventional conditions, with the temperature at 21–23 °C, humidity at 51–54%, and in a 12-h light/dark cycle with food and water supplied ad libitum. To induce liver injury, CCl₄ (Sigma-Aldrich, St. Louis, MO, USA) was prepared as a 10% solution in mineral oil (Sigma-Aldrich), and a volume equivalent to 10 μ L/g of the mouse body weight was injected into the intraperitoneal cavity twice per week for 3 weeks. In the fourth week, 200 μ L of concentrated T-MSC CM or 1 μ g rhIL-1Ra (Peprotech, Rocky Hill, NJ, USA) was injected twice via the tail vein,

and the mice were sacrificed 2 weeks later. An illustration of the experimental design is shown in Fig. 1.

2.5 Hematoxylin and eosin (H&E) staining

Mouse livers were isolated and fixed with 4% PFA solution in PBS. Fixed tissues were embedded in paraffin and sectioned at 5- μ m thickness, and then subjected to H&E staining. Fields were taken using the Olympus DP71 and the DP controller software (Tokyo, Japan) and used for histological analysis.

2.6 Picro-Sirius Red staining

For the visualization of collagen accumulation Picro-Sirius Red staining was performed using the Picro-Sirius Red Stain Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, paraffin-embedded tissue sections were hydrated and incubated in the staining solution for 60 min. Slides were rinsed twice in acetic acid solution, and once in absolute alcohol. Dehydration and mounting of the slides were performed, and then fields were determined. The pixel densities of digital images were measured from more than 10 different fields using ImageJ software (<https://imagej.nih.gov/>).

2.7 RNA extraction and cDNA synthesis

RNA extraction was performed using the TRIzol reagent (Thermo Fisher Scientific), and 1 μ g of total RNA was used for the synthesis of cDNA using the 5 $\times$ Reverse Transcription Master Premix (ELPIS-Biotech, Daejeon, Korea).

2.8 Real-Time quantitative PCR

For the quantitation of gene expression, a primer pair (0.4 μ M) and the reagent from the SensiFAST SYBR Hi-ROX Kit (Bioline, London, UK) were mixed with the prepared cDNA. The following primer sequences were used for the amplification; 5'-GCTCCTCTTAGGGGC-CAC-3' and 5'-CCACGTCTCACCATTGGGG-3' for Col1a1; 5'-CTCCCGTGGCTTCTAGTGC-3' and 5'-

Fig. 1 Schema of the experimental design. The timeline of the experiment is as indicated. Liver injury was induced via six intraperitoneal (i.p.) injections of CCl₄ prepared in mineral oil.

T-MSC CM or rhIL-1Ra was injected intravenously (i.v.) twice, and mice were sacrificed 2 weeks later

Fig. 2 Characterization of T-MSCs in culture. **A** Analyses of surface marker expression using flow cytometry. The light gray histogram represents the isotype control, and the dark gray represents the specific antibody. **B** Plastic adherence and mesodermal differentiation of T-MSCs were demonstrated under phase-contrast microscopy

($\times 100$ magnification for lanes plastic adherence, osteogenic, $\times 200$ magnification for lanes adipogenic, chondrogenic). Oil Red O, Alcian Blue, and Alizarin Red S staining were performed after 3 weeks of culture in adipogenic, chondrogenic, and osteogenic differentiation medium, respectively

GCCTTAGTTTGGACAGGATC-3' for Tgfb1; 5'-CTTGGTTCCCTGGCGTACTC-3' and 5'-ACCTGATCCGTCCACAAACAG-3' for Timp1; 5'-CAAGTTCCCCGGCGATGTC-3' and 5'-TTCTGGTCAAGGTCACCTGT-3' for Mmp2; 5'-CTGGACAGCCAGACACTAAA-3' and 5'-CTCGCGGCAAGTCTTCAGAG-3' for Mmp9; 5'-ACTGCCAGAAGAGGCACTCC-3' and 5'-CGATCACCCCGAAGTTCA-3' for Tnfa; 5'-AGTTGCCTTCTTGGGACTGA-3' and 5'-CAGAATTGCCATTGCACAAC-3' for Il6; 5'-GCAACTGTTGCTGAACTCAACT-3' and 5'-ATCTTTTGGGGTCCGTCAACT-3' for Il1b; 5'-AGGTCGGTGTGAACGGATTT-3' and 5'-TGTAGACCATGTAGTTGAGG-3' for GAPDH as a housekeeping gene. Amplification was performed using 40 cycles of a 15 s denaturation step at 95 °C and a 1-min amplification and signal acquisition step at 60 °C, using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Cycle threshold (Ct) values were obtained, and the relative expression level of the target gene was determined as $2^{-(\text{Gapdh Ct} - \text{target gene Ct})}$.

2.9 Statistical analysis

Student's *t* test or one-way analysis of variance with Tukey's multiple comparisons test was performed for statistical analysis using GraphPad Prism 7.03 (GraphPad Software, La Jolla, CA, USA). Data are presented as the mean $\pm$ standard error of the mean (SEM). We considered $p < 0.05$ to indicate a statistically significant difference.

3 Results

3.1 Phenotype and differentiation potential of T-MSCs

T-MSCs at a proliferating stage were harvested and examined for the expression of surface markers. Data showed that T-MSCs are negative for hematopoietic stem cell markers (CD34 and CD45) and positive for primitive cell markers (CD73 and CD109) (Fig. 2a). The differentiation potential of T-MSCs into mesodermal origin was also evaluated by culturing the cells using differentiation kits. After 3 weeks, adipogenic differentiation was examined by lipid droplet formation, chondrogenic differentiation by glycosaminoglycan staining, and osteogenic differentiation by matrix mineralization. The results demonstrated the multipotent differentiation of T-MSCs (Fig. 2b).

3.2 Amelioration of liver inflammation by T-MSC CM or rhIL-1Ra treatment

To determine if T-MSC CM or rhIL-1Ra treatment could resolve liver injury induced by CCl₄ treatment, H&E staining was performed using paraffin-embedded liver samples. Increased portal and lobular inflammation, as well as hepatocyte necrosis, was detected in CCl₄-injured mouse liver samples, and treatment with T-MSC CM or rhIL-1Ra resulted in a reduction in liver inflammation (Fig. 3a). Histological scores of inflammation and necrosis were

Fig. 3 T-MSC CM or rhIL-1Ra treatment ameliorated liver inflammation. **A** Representative images of H&E staining from mouse livers ($\times 100$ magnification in upper panel; $\times 200$ magnification in lower panel). **B** Evaluation criteria for the scoring of liver inflammation.

evaluated according to the criteria given in Fig. 3b [14]. The results showed a sixfold increase in histological score upon CCl_4 treatment, whereas T-MSC CM or rhIL-1Ra treatment significantly reduced the scores representing liver inflammation and necrosis (Fig. 3c).

3.3 Resolution of liver fibrosis by T-MSC CM or rhIL-1Ra treatment

Next, we examined the occurrence of hepatic fibrosis upon CCl_4 injection and its resolution by T-MSC CM or rhIL-1Ra treatment. Picro-Sirius Red staining was performed, and the accumulation of collagen fibers was visualized. As shown in Fig. 4a, CCl_4 injection enhanced collagen deposition and resulted in liver fibrosis, and this was

significantly reduced by T-MSC CM or rhIL-1Ra treatment. Quantitation of collagen deposition showed an increase in collagen accumulation in the CCl_4 -treated group of mice, and its decrease upon T-MSC CM or rhIL-1Ra injection (Fig. 4b). The expression levels of the genes involved in fibrosis, alpha-1 type I collagen (COL1A1), and TGF- β 1, were determined by real-time qPCR. In agreement with the histological analysis, mRNA expression of both COL1A1 and TGF- β 1 was highly upregulated on CCl_4 injection, and significantly reduced following T-MSC CM or rhIL-1Ra treatment (Fig. 4c).

Fig. 4 T-MSC CM or rhIL-1Ra treatment reduces liver fibrosis. **A** Representative images of Picro-Sirius Red staining from mouse livers ($\times 200$ magnification). **B** Pixel densities of digital images were measured and compared between groups. **C** Expression of the genes

involved in fibrosis, COL1A1 and TGF- β 1, was measured from whole liver extracts and compared between groups. Data are presented as mean $\pm$ SEM ($n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.4 Changes in the expression of fibrosis and inflammatory markers

It is well documented that the upregulation of tissue inhibitor of metalloproteinase protein 1 (TIMP1) expression is involved in the development of liver fibrosis [15]. We examined mRNA expression of TIMP1 in the mouse liver and found it to be increased more than 70-fold upon CCl₄-induced liver injury; these levels decreased on T-MSC CM or rhIL-1Ra treatment to levels similar to those seen in the normal control mouse group. However, no significant changes in the expression levels of two gelatinases, MMP-2 and MMP-9, were determined among the experimental groups (Fig. 5a). This might indicate that amelioration of liver fibrosis by T-MSC CM or rhIL-1Ra treatment was partly due to the downregulation of TIMP1 expression, which resulted in an increase in the activity of matrix metalloproteinases. Next, we investigated the expression levels of inflammatory molecules in liver specimens by real-time qPCR. Expression of TNF- α was significantly upregulated by CCl₄ treatment, and T-MSC CM or rhIL-1Ra treatment decreased the expression levels of TNF- α in the mouse liver. Changes in IL-6 or IL-1 β expression did

not show statistical significance, but the trend was the same as that seen in TNF- α (Fig. 5b).

4 Discussion

This study demonstrates the therapeutic potential of T-MSC CM in the resolution of liver fibrosis. CCl₄ was used to induce liver injury in mice, and T-MSC CM or rhIL-1Ra was injected to determine their therapeutic potential. Our results suggest that IL-1Ra is an effective molecule that is exclusively secreted by T-MSCs [3] and can exert anti-inflammatory and anti-fibrotic effects in the CCl₄-injured liver. The regulation of inflammation by treatment with T-MSC CM or IL-1Ra was demonstrated by gross histological analysis of liver specimens and measurement of TNF- α expression. Resolution of fibrosis was determined by a reduction in collagen deposition and a decrease in the expression of COL1A1, TGF- β 1, and TIMP1.

Previously, we demonstrated that T-MSCs produce an anti-inflammatory cytokine, IL-1Ra, at levels much higher than those seen in BM-MSCs or AD-MSCs [3].

Fig. 5 T-MSC CM or rhIL-1Ra treatment regulates ECM-modulating and proinflammatory genes. The mRNA expression levels of genes involved in **A** ECM modulation (TIMP1, MMP2, and MMP9),

and **B** inflammation (TNF- α , IL-6, and IL-1 β) were measured and compared between experimental groups. Data are presented as mean $\pm$ SEM (n = 3, * p < 0.05, ** p < 0.01)

Semiquantitative analysis of T-MSC CM has revealed that approximately 100 ng of IL-1Ra is secreted from 2×10^5 T-MSCs. We reported that IL-1Ra secreted from T-MSCs affects fibroblast migration toward injured myotubes and inhibits proliferation and fibronectin production in these cells, thereby preventing muscle fibrosis. IL-1Ra is an endogenous receptor antagonist that blocks IL-1 signaling, resulting in promotion of the anti-inflammatory response and maintenance of immunological homeostasis [8]. Mice lacking the IL-1 receptor have been shown to be protected from liver cirrhosis [10]; here we demonstrate the protective role of IL-1Ra in liver inflammation and fibrosis.

The multipotency of MSCs opens up a multitude of uses for these cells in the treatment of diseases involving tissue regeneration. Resolution of liver cirrhosis upon MSC transplantation has been reported by many different groups [16–18], and we have demonstrated that T-MSCs migrate to the injured organ and differentiate into hepatocytes [6]. However, increasing the number of hepatocytes alone would have limitations in rehabilitating damaged tissue when its architecture is already altered, and fibrotic triggers

are still present. Therefore, the simultaneous modulation of nonparenchymal cells would serve as an effective therapeutic strategy. Here we dissociated the paracrine effects of T-MSCs from multipotency in the amelioration of liver fibrosis. Although it is not directly compared, T-MSC CM treatment alone demonstrated therapeutic effects without the cells. This suggests the usefulness and possibilities inherent in developing a cell-free therapy using MSCs. Further studies may investigate the effects of T-MSC CM or candidate molecules (i.e. IL-1Ra) on the regulation of hepatocyte survival and/or death, as well as the activation of nonparenchymal cells, including HSCs and Kpffer cells.

Fibrosis is defined as an end-stage pathological symptom in several organs, including the liver, skin, heart, kidney, and lung. Although there is a range of organ-specific triggers that induce tissue damage, the fibrotic process and subsequent signaling pathways are common in injured organs [19]. This suggests the possibility that a universal anti-fibrotic agent can be developed. Pirfenidone, a modulator of TGF- β 1 activity, is an approved drug for the

treatment of idiopathic pulmonary fibrosis, and its efficacy in the resolution of organ fibrosis has been reported in various animal models [20–22]. However, several adverse effects have been reported, and there is a growing need for multiple drugs to treat fibrosis. The effectiveness of MSC injection in the reduction of organ fibrosis has been widely explored [23, 24] and the secretion of IL-1Ra from T-MSCs supports the idea of developing a novel therapy using MSCs.

In summary, we demonstrated the paracrine effects of T-MSCs in the resolution of liver fibrosis by reducing tissue inflammation and collagen deposition. The efficacy of IL-1Ra treatment was determined in parallel and showed that the therapeutic effect of T-MSC CM is similar to the anti-fibrotic agent, IL-1Ra. These findings further strengthen the therapeutic potential of T-MSCs for the treatment of fibrosis in multiple organs, and the usefulness of T-MSC CM for the identification of novel, endogenous anti-fibrotic molecules.

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Compliance with ethical standards

Conflicts of interest The authors declare no actual or potential conflict of interest.

Ethical statement All experimental procedures and protocols were approved by the Animal Ethics Committee at Ewha Womans University College of Medicine (Seoul, Korea; No. ESM 18-0405), and experiments were performed in accordance with relevant guidelines and regulations.

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