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Therapeutic potential of human adipose tissue-derived multi-lineage progenitor cells in liver fibrosis

Hanayuki Okura [a,b], Mayumi Soeda [a], Mitsuko Morita [a], Maiko Fujita [a], Kyoko Naba [a], Chiyoko Ito [a], Akihiro Ichinose [c], Akifumi Matsuyama [a,b,*]

[a] Platform of Therapeutics for Rare Disease, National Institute of Biomedical Innovation, 5-5-2-602 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan
[b] The Center for Medical Engineering and Informatics, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0879, Japan
[c] Department of Plastic Surgery, Kobe University Hospital, 7-5-2 Kusunoki-cho, Chuo-ku, Kobe, Hyogo, Japan

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ABSTRACT

Introduction: Liver fibrosis is characterized by excessive accumulation of extracellular matrix. In a mouse model of liver fibrosis, systemic injection of bone marrow mesenchymal stem cells (BM-MSCs) was considered to rescue the diseased phenotype. The aim of this study was to assess the effectiveness of human adipose tissue-derived multi-lineage progenitor cells (hADMPCs) in improving liver fibrosis.
Methods and results: hADMPCs were isolated from subcutaneous adipose tissues of healthy volunteers and expanded. Six week-old male nude mice were treated with carbon tetra-chloride (CCl_4) by intraperitoneal injection twice a week for 6 weeks, followed by a tail vein injection of hADMPCs or placebo control. After 6 more weeks of CCl_4 injection (12 weeks in all), nude mice with hADMPCs transplants exhibited a significant reduction in liver fibrosis, as evidenced by Sirius Red staining, compared with nude mice treated with CCl_4 for 12 weeks without hADMPCs transplants. Moreover, serum glutamic pyruvate transaminase and total bilirubin levels in hADMPCs-treated nude mice were lower levels than those in placebo controls. Production of fibrinolytic enzyme MMPs from hADMPCs were examined by ELISA and compared to that from BM-MSCs. MMP-2 levels in the culture media were not significantly different, whereas those of MMP-3 and -9 of hADMPCs were higher than those by BM-MSCs.
Conclusion: These results showed the mode of action and proof of concept of systemic injection of hADMPCs, which is a promising therapeutic intervention for the treatment of patients with liver fibrosis.

1. Introduction

Various conditions such as viral hepatitis, chronic alcohol abuse, metabolic diseases, autoimmune diseases and bile duct epithelial injury can cause liver fibrosis [1,2]. Liver fibrosis is reversible, whereas cirrhosis, the end-stage result of fibrosis, is in general irreversible [3]. Liver fibrosis is characterized by excessive accumulation of extracellular matrix, with the formation of scar tissue encapsulating the area of injury [4]. The prognosis of patients with liver fibrosis is poor, but liver transplantation seems to improve the prognosis [5,6]. However, limited numbers of donor livers are available for the millions of patients who need them worldwide [7]. Thus, there is a need for novel therapeutic approaches.

Recently, cell therapy has been proposed as an attractive tool for treatment of patients with severe liver disease [8–13]. Stem/progenitor cells, which possess certain characteristics including self-renewal, proliferation, longevity, and differentiation, are valuable in cell therapy [14]. Several groups have demonstrated the effectiveness of bone marrow-derived mesenchymal stem cells (BM-MSCs) in animal models of liver fibrosis and cirrhosis [15–18]. However, others have reported the lack of any changes in the extent of liver fibrosis or liver function tests following the use of BM-MSCs in a rat model of severe chronic liver injury [19]. Thus, the therapeutic efficacy of BM-MSCs transplantation remains controversial at present [19].

Adipose tissue-derived progenitor/stem cells are an attractive cell source for cell therapy of liver fibrosis, based on several properties of these cells; (1) ample production of fibrinolytic enzymes and cytokines [20], (2) ease of obtaining stem cells compared to other tissue-specific stem cells including BM-MSCs, [21]. The use of human adipose tissue-derived multi-lineage progenitor cells (hADMPCs) supports the view that cytokine production could mediate the therapeutic actions of hADMPCs in liver fibrosis.

* Corresponding author at: Platform of Therapeutics for Rare Diseases, National Institute of Biomedical Innovation, 5-5-2-602 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan. Fax: +81 78 304 6176.
E-mail address: akifumi-matsuyama@umin.ac.jp (A. Matsuyama).

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In the present study, we investigated the efficacy of treatment using hADMPCs in nude mice with CCl_4-induced chronic liver dysfunction and the mechanism of their action in improvement of liver fibrosis.

2. Materials and methods

2.1. Adipose tissue

Adipose tissue samples were resected from 7 human subjects during plastic surgery (all females, age, 20–60 years) as excess discards. About 10–50 g subcutaneous adipose tissue was collected from the sample of each subject. All subjects provided informed consent. The protocol was approved by the Review Board for Human Research of Kobe University, Graduate School of Medicine, Osaka University, Graduate School of Medicine and National Institute of Biomedical Innovation, Japan.

2.2. Isolation and expansion of hADMPCs

hADMPCs were prepared as described previously [8–10]. Briefly, the resected excess adipose tissue was minced and then digested at 37 °C for 1 h in Hank's balanced salt solution (HBSS, GIBCO Invitrogen, Grand Island, NY) with Liberase (Roche Diagnostics, Germany) as indicated by the manufacturer. Digests were filtered through a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at 800×*g* for 10 min. Red blood cells were excluded using density gradient centrifugation with Lymphoprep (d = 1.077; Nycomed, Oslo, Norway), and the remaining cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen) with 10% defined fetal bovine serum (FBS, Biological Industries, Israel) for 24 h at 37 °C. Following incubation, the adherent cells were washed extensively and then treated with 0.2 g/l ethylenediaminetetraacetate (EDTA) solution (Nacalai Tesque, Kyoto, Japan). The resulting suspended cells were replated on retronectin (RN)-coated dishes (Takara, Kyoto, Japan) in SteMedis (Nipro, Osaka, Japan), 1× insulin-transferring selenium (Nipro, Osaka), 1 nM dexamethasone (MSD, Tokyo, Japan), 100 μM ascorbic acid 2-phosphate (Sawai Pharmaceuticals Co., Osaka), 10 ng/ml epidermal growth factor (EGF, PeproTec, Rocky Hill, NJ), and 5% FBS (FBS, Biological Industries, Israel). The culture medium was changed twice a week and then the cells were applied for the experiments after 5–6 passages.

2.3. Flow cytometric analysis of hADMPCs

hADMPCs were characterized by flow cytometry. Cells were detached and stained with anti-human CD31, CD34, CD44, CD45, CD56, CD73, CD90, CD105 or CD166 antibodies (BD Lyoplate™ Screening Panels, BD Bioscience, San Jose, CA). Isotype-identical antibodies served as controls. After washing with Dulbecco's phosphate-buffered saline (PBS, Nacalai Tesque), cells were incubated with PE-labeled goat anti-mouse Ig antibody (BD PharMingen) for 30 min at 4 °C. After three washes, the cells were resuspended in PBS and analyzed by flow cytometry using a guava easyCyte flow cytometry systems (Merck Millipore, Darmstadt, Germany).

2.4. Adipogenic, osteogenic and chondrogenic differentiation procedure

Tri-lineage differentiation was examined as described previously [22]. Briefly, for adipogenic differentiation, the cells were cultured in Differentiation Medium (Zen-Bio, Inc.). After three days, half of the medium was replaced with Adipocyte Medium (Zen-Bio, Inc.) every two days. Five days after differentiation, characterization of adipocytes was confirmed by microscopic observation of intracellular lipid droplets after Oil Red O staining. Osteogenic differentiation was induced by culturing the cells in DMEM containing 10 nM dexamethasone, 50 mg/dl ascorbic acid 2-phosphate, 10 mM β-glycerophosphate (Sigma), and 10% FBS. Differentiation was examined by Alizarin red staining. For chondrogenic differentiation, 2×10^5 cells of the hADMPCs were centrifuged at 400×*g* for 10 min. The resulting pellets were cultured in chondrogenic medium (α-MEM supplemented with 10 ng/ml TGF-β, 10 nM dexamethasone, 100 M ascorbate, and 10 μl/ml 100× ITS Solution) for 14 days. For Alcian Blue staining, nuclear counter-staining with Weigert's hematoxylin was followed by 0.5% Alcian Blue 8GX for proteoglycan-rich cartilage matrix.

2.5. Animal model of liver fibrosis and cell administration

Chronic liver fibrosis was induced in nude mice using the procedure described previously [23,24] with some modification. Briefly, 6-week-old male nude mice (body weight of 20–30 g purchased from CLEA, Tokyo) were treated with a mixture of CCl_4 (Wako Pure Chemicals, Osaka) (0.3 ml/kg) and olive oil (Wako Pure Chemicals) (1:1 vol/vol) by intra-peritoneal injection twice a week for 6 weeks, and this was followed by a tail vein injection of hADMPCs (1.0×10^6 cells/kg body weight, n = 4) or placebo control (n = 5), and followed by 6 more weeks of CCl_4 treatment.

2.6. Liver function tests and histological analysis

Blood specimens were collected by cardiac puncture at the end of the experiment. Measurement of serum albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total-bilirubin levels by routine laboratory methods was outsourced to Oriental Yeast Co. (Shiga, Japan).

Hematoxylin and eosin (H&E) staining and Sirius Red (SR) staining were performed to determine the extent of liver inflammation and fibrosis. The stained slides were viewed on a BioZero laser scanning microscope (Keyence, Osaka). The area of liver fibrosis was quantified with SR staining. Briefly, the fibrotic area (red staining) was assessed at 40× magnification using computer-assisted image analysis with All-in-One analysis software (Keyence, Osaka). Sixteen fields were randomly selected for each group.

2.7. Measurement of MMP-2, -3 and -9 production by hADMPCs

One million cells of hADMPCs and BM-MSCs (DS Pharma Biomedical, Osaka) were seeded onto 6 well plates and then cultured for 24 h. The supernatants were harvested, centrifuged, and frozen at −80 °C until analysis. MMP-2, MMP-3 and MMP-9 were measured by enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, MN) using the instructions supplied by the manufacturer.

2.8. Statistical analysis

Serum parameters and fibrotic area are presented as mean ± SD. Differences between groups were assessed for statistical significance by the Student's t-test, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Characterization of hADMPCs

Flow cytometry was used to assess markers expressed by hADMPCs (Fig. 1A). The cells were negative for markers of

hematopoietic lineage (CD45) and hematopoietic stem cells, CD34 and CD133. They were also negative for CD31, an endothelial cell-associated marker, and c-Kit (CD117), a cell surface antigen. However, they stained positively for several surface markers characteristic of mesenchymal stem cells, but not embryonic stem (ES) cells, such as CD29, CD44 (hyaluronan receptor), CD73 and CD105 (endoglin).

Next, we examined the adipogenic, osteogenic and chondrogenic differentiation potentials of hADMPCs. Adipogenic differentiation was confirmed by accumulation of intracellular lipid droplets stained with Oil Red O (Fig. 1B). Differentiation and induction of hADMPCs was associated with increase in the amount of Oil Red O-stained lipid droplets, indicating that hADMPCs can differentiate into adipocytes. Osteogenic induction was examined by Alizarin red S staining (Fig. 1B). Induction of hADMPCs for osteogenesis was associated with Alizarin red S staining and appearance of mineralized nodules. The chondrogenic potential of hADMPCs is shown in Fig. 1B. Induction of chondrogenesis by pellet culture resulted in staining of extracellular matrices of hADMPCs-derived pellet-cultured chondrocytes for Alcian Blue, indicating the chondrogenic differentiation potential of hADMPCs. These results confirmed the tri-lineage differentiation potential of hADMPCs and the mesenchymal stem cell properties of hADMPCs.

3.2. Effects of hADMPC on CCl_4-induced chronic liver dysfunction in nude mice

We adopted the CCl_4-induced chronic mouse fibrosis model in this study rather than the CCl_4-induced acute model because CCl_4-induced acute liver fibrosis resolves spontaneously [25]. For this purpose, 9 male nude mice were injected intraperitoneally with CCl_4 twice weekly for 6 weeks, and then divided into two groups, 4 animals received hADMPCs transplantation via the tail vein and the other 5 vehicle control received Ringer's solution with 1/30 volume of heparin. All animals were followed for 6 weeks after the last injection (a).

H&E staining of liver sections showed reduced hepatocyte vacuolar degeneration in hADMPC-transplanted CCl_4-injured mice compared with the control (Fig. 2B). The peri-lobular regions were the main areas affected by CCl_4 hepatotoxicity while the centrilobular regions seemed to be the least affected. These findings suggest intact albumin secretion, which was confirmed by Sirius Red (SR) staining of control liver sections. SR staining of sections from hADMPC-transplanted mice showed mild liver fibrosis, while that of sections from control group mice showed moderate fibrosis (Fig. 2B). Quantitative image analysis of the fibrotic area in SR-stained sections confirmed the efficacy of hADMPC-transplantation on liver fibrosis. The mean fibrotic area was significant lower in hADMPC-transplanted CCl_4-injured mice ($1.8 \pm 1.1\%$ of fibrotic areas) than control mice ($10.9 \pm 3.9\%$ of fibrotic areas) ($p < 0.05$), indicating that hADMPC-transplantation ameliorated liver fibrosis and increased the area containing hepatocytes (c).

3.3. Functional recovery of liver damage following transplantation of hADMPCs

We next evaluated the effects of cell transplantation on the extent of liver injury and liver function. Serum transaminase levels (AST and ALT) were significantly higher in mice with liver damage (control), but the increase was attenuated by hADMPCs transplantation (Fig. 3A and B). These results confirmed the effectiveness of hADMPCs in the treatment of liver damage associated with fibrosis.

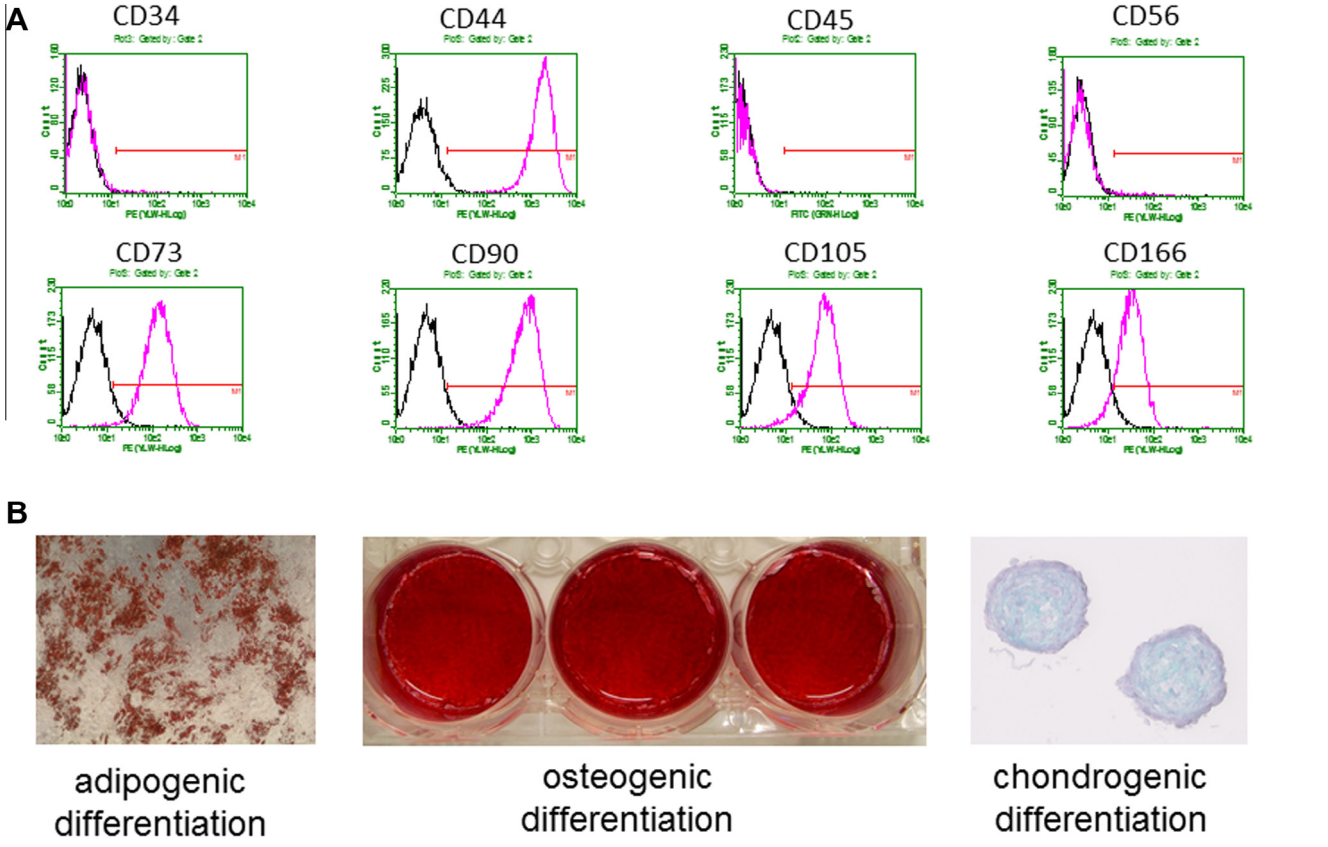

Fig. 1. Characterization of hADMPCs. (A) Flow cytometric characterization of hADMPCs. (B) An isotype-matched negative control indicated as red curve. (C) Adipogenic, osteogenic and chondrogenic differentiation potentials of hADMPCs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

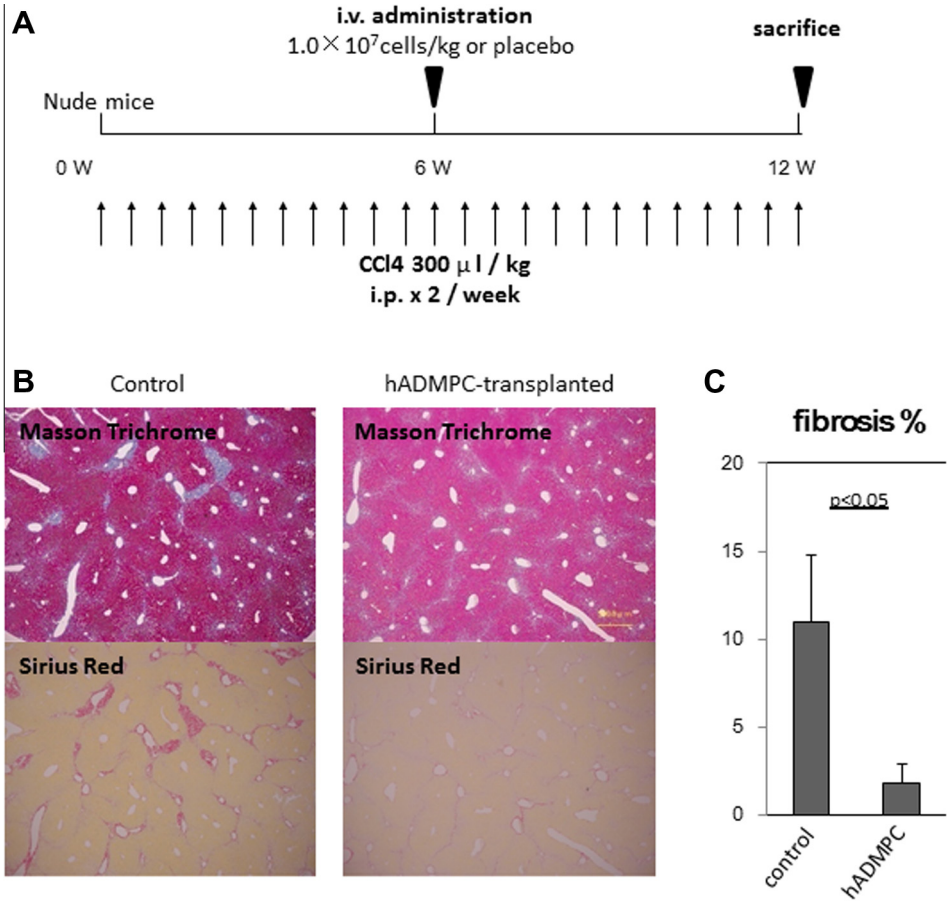

Fig. 2. Assessment of liver fibrosis in hADMPC-transplanted nude mice and controls. (A) Diagram of the treatment protocol. (B) Extracellular deposition of collagen fibers stained with Sirius Red. (C) Quantification of collagen by image analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Interestingly, serum albumin level remained high after hADMPCs, similar to the control (Fig. 3C). These results could be explained by damage of the centrilobular region, the main site of albumin production. Considered together, the results suggest the beneficial effects of hADMPCs in attenuating liver damage and recovery of liver function.

3.4. hADMPCs-induced functional recovery is mediated by MMP release

Finally, we analyzed the mechanism of the hepatoprotective effect of hADMPCs. For this purpose, we measured the amount of the fibrinolytic enzymes, MMP-2, MMP-3 and MMP-9, secreted by hADMPCs by ELISA (Fig. 4). After 3-day culture, the amounts of enzymes production by hADMPCs and BM-MSCs were measured. There was no significant difference in MMP-2 production by hADMPCs and BM-MSCs (59.7 ± 2.3 vs 58.3 ± 0.0 ng/ml from 1.0×10^4 cells cultured for 3 days). On the other hand, MMP-3 and MMP-9 production levels were significantly higher in hADMPCs than BM-MSCs (6.84 ± 2.3 vs 0.03 ± 0.0 ng/ml, $p < 0.05$, 0.462 ± 0.015 vs 0.003 ± 0.008 ng/ml, $p < 0.05$, from 1.0×10^4 cells cultured for 3 days, respectively).

4. Discussion

The major finding of the present study was improvement of liver fibrosis in CCl_4-induced mice after systemic administration of hADMPCs, and that this effect was mediated, at least in part, through the production of fibrinolytic MMP-2, -3, and -9, from hADMPCs, suggesting that these cells could be particularly effective in resolving liver fibrosis.

Liver transplantation is an established treatment for severe liver cirrhosis, although the number of patients who could benefit from such treatment is small due to the limited number of donors [5]. Cell therapy has been proposed as an alternative and attractive tool for treating patients with severe liver disease [8–13]. Among the cell therapy tested so far, hepatocyte replacement therapy had been examined. Isolated hepatocytes from human liver [13], regenerated hepatocyte-like or –progenitor cells from embryonic, induce pluripotent [26,27], or hepatic progenitor cells [11], and *in situ* reprogrammable cells (9, 10) have been tested for their efficacy in animal models. The strategy has also been successful in clinical trials involving patients with certain inherited diseases [28]. Although large numbers of hepatocytes or hepatocyte-like cells are needed for meaningful cure and there should be no room for the cells in fibrotic hepatic parenchyma to engraft, such replacement therapies, however, do not seem to be clinically fruitful for liver fibrosis. We hypothesized that fibrolytic enzymes produced by hADMPCs could be useful for treatment of liver fibrosis, and therefore shifted the treatment strategy to improvement of liver fibrosis with cell-based fibrinolytic enzymes delivery. In this strategy, hADMPCs derived-MMPs should produce lysis of excess extracellular matrices and make room for the patient's own proliferative hepatocytes.

To establish the cell-based fibrinolytic enzyme delivery therapy as first line next to liver transplantation, some challenging issues

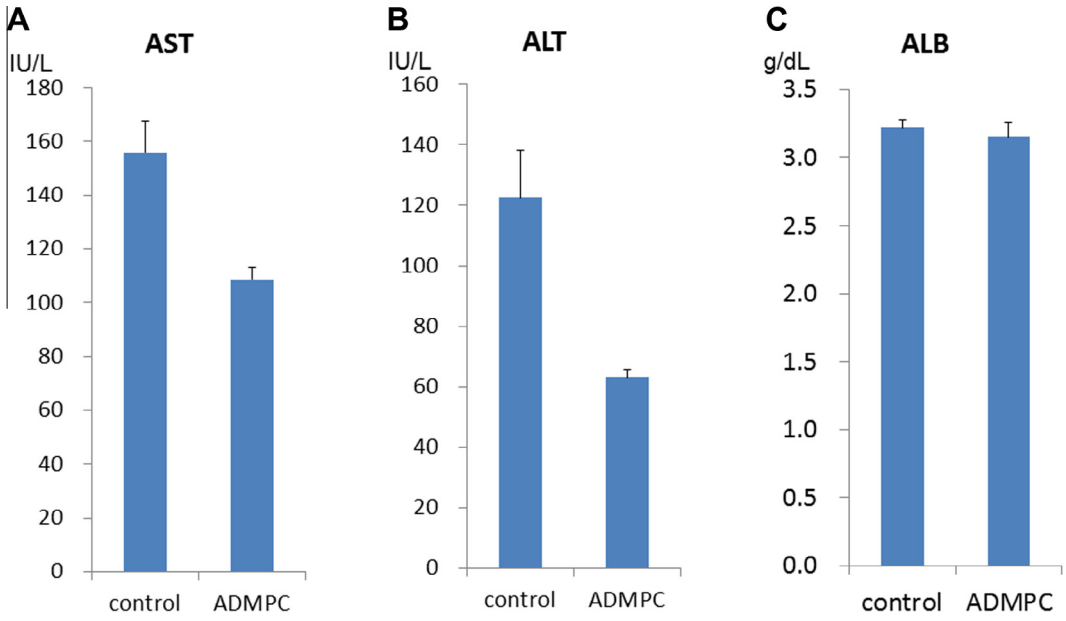

Fig. 3. Examination of serum parameters. (A, B) Transaminase (AST and ALT), (C) Serum albumin. Data are mean ± SD. C: control mice; T: mice transplanted mice with hADMPCs.

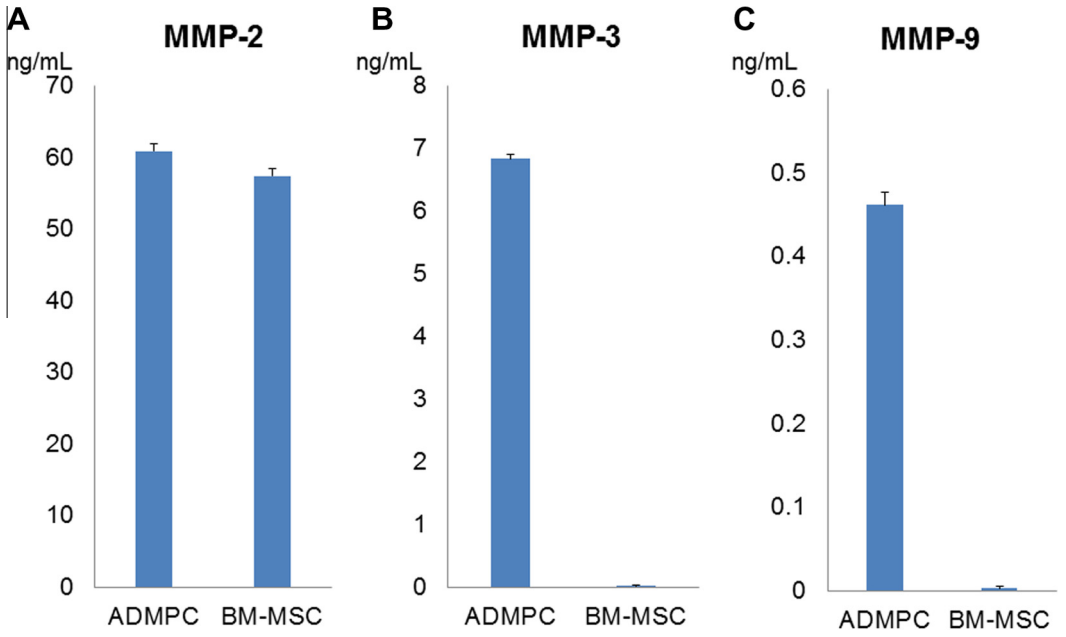

Fig. 4. Quantitative analysis of MMP-2, MMP-3 and MMP-9 level produced by hADMPCs and BM-MSCs. The amount of MMP-2 (A), MMP-3 (B) and MMP-9 (C) after 3 days of culture.

should be dealt with; (1) the cells should be obtained easily and ethically in large quantities, (2) the cells should improve liver fibrosis and liver panel, and (3) the cells act as vehicle for the delivery of MMPs.

The first issue is whether the cells could be obtained easily and ethically in large quantities. hADMPCs is favorable for the therapy because adipose tissue, from which ADMPCs are obtained, is easily and safely accessible and large quantities of the tissues can be obtained without serious ethical issues, since liposuction surgery yields from 100 ml to >3 L of lipoaspirate tissue [8–10]. Therefore, hADMPCs can potentially be applied not only for autologous but also allogenic cell-based enzyme delivery in the future. Based on the above advantages, hADMPCs represent a potentially promising source of cells for the therapy.

Second, we need to show that hADMPCs-administration results in improvement of liver fibrosis and liver panel, as proof-of-concept of therapy. As shown in Fig. 2, hADMPC significantly improved liver fibrosis in CCl_4-treated nude mice (a model of chronic liver cirrhosis). The treatment also resulted in improvement of serum transaminase levels. In this model, massive fibrosis was mainly noted in the peri-hepatic lobular region but not in the centrilobular regions surrounding the central veins. Albumin is known to be mainly produced by hepatocytes in the centrilobular region. This is the most likely reason for the lack of difference in serum albumin

levels between hADMPC-transplanted animals and controls. These results indicate that hADMPCs transplantation showed the proof-of-concept to liver dysfunction associated with fibrosis.

Finally, an important issue in this kind of therapy is whether the cells secrete sufficient amount of MMPs. One study reported that matrix metalloproteinase gene delivery could decrease collagen fibers and reduce liver fibrosis [29]. The mode of action was considered to be the strong expression MMPs on the transplanted cells, indicating that MMPs-producing cells other than BM-MSCs [30] are suitable for use for the cell-based enzyme delivery. The present study showed that hADMPCs expressed MMP-2, -3 and -9 (Fig. 2). There was no significant difference in MMP-2 production between hADMPC and BM-MSCs. However, the production of MMP-3 and MMP-9 from hADMPCs was superior to that from BM-MSCs. MMP-3 and MMP-9 are known to lyse collagen types III and I, which are major compartment of liver fibrotic lesion [29]. These data highlight the potential effectiveness of hADMPCs in the treatment of liver fibrosis and the superiority of hADMPCs compared to other therapies.

In conclusion, the present study demonstrated that systemic administration of hADMPCs significantly attenuated liver fibrosis and improved liver function, and that the therapeutic effect of hADMPCs was in part due the secretion of fibrinolytic enzymes, MMPs. These proofs of concept and mode of action prompted us to choose hADMPCs for cell therapy of liver fibrosis. hADMPCs therapy, as cell-based enzyme delivery therapy, has the potential to be an effective source of inducers that support liver regeneration.

Declaration

The authors declare no conflict of interest.

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