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

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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 enzymes MMPs from hADMPCs was 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 [20]. Thus, the therapeutic efficacy of BM-MSCs transplantation remains controversial at present [20].

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 [21], (2) ease of obtaining stem cells compared to other tissue-specific stem cells including BM-MSCs, [22]. 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.

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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 [23]. 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 [24,25] 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

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