Cell Therapy Using Adipose-Derived Stem Cells for Chronic Liver Injury in Mice

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The present study investigated whether transplantation of autologous adipose-derived stem cells (ASCs) administered into the systemic circulation of a mouse with chronic liver injury provides therapeutic efficacy in the absence of any undesirable side effects. The ASCs used were isolated from mice with the same genetic background as the recipient mice and expanded in vitro. For the induction of chronic liver injury, mice were repetitively administered twice a week with CCl₄, a well-known hepatotoxin, for a period of 4 weeks. One day after the eighth dose of CCl₄, ASC transplantation was performed by tail vein injection and subsequently followed by two additional doses of CCl₄ administration. The recipient mice were divided into four groups (vehicle control, 1.5×10³, 1.5×10⁴, and 1.5×10⁵ ASCs per mouse). One day after the final CCl₄ administration, all mice were sacrificed to assess serum markers and liver histology. The level of serum markers for liver injury and hepatic function did not differ among the four groups. Similarly, no difference was observed in the liver histology between groups. Cell transplantation with ASCs in our model of chronic liver failure did not result in any observable side effects, but from our results, a single application of ASCs seems to be ineffective in improving liver injury.

Key words: Cell transplantation; Adipose-derived stem cells; Chronic liver injury; Liver; Animal model

INTRODUCTION

Chronic liver diseases follow a common pathological course from mild to severe inflammation followed by progression to the development of fibrosis. Ultimately, these livers become cirrhotic, which is an irreversible fatal stage of end-stage liver disease (24). The pathology of fibrosis and liver cirrhosis are a consequence of being in a sustained state of chronic liver injury. To prevent liver diseases from progressing toward liver cirrhosis, the development of new therapeutic approaches applicable to the various stages of chronic liver diseases are needed. A widely used animal model to investigate pathological processes involved in acute and chronic liver injuries as well as liver cirrhosis is mice treated with repetitive injections of carbon tetrachloride (CCl₄).

Cell-based therapies using bone marrow or stem cells have been a recent area of intense investigation in the field of liver regenerative medicine. Compared with whole-liver organ transplantation, cell-based transplantation procedures are minimally invasive and results in few complications. Indeed, the infusion of bone marrow or embryonic stem

cells has led to the restoration of normal function in fibrotic livers (5,14,22). To date, the most optimal source of autologous and transplantable cells remains to be determined, but cumulative data suggest that adipose-derived stem cells (ASCs) may be attractive due to several factors: first, the abundant number of isolatable adult stem cells in the adipose tissue; second, the easy access of the adipose tissue; and third, the effectiveness of ASCs in the treatment of various inflammatory diseases, including acute liver inflammation (1,8,30). Moreover, ASCs can secrete a variety of cytokines and/or enzymes and has the therapeutic potential to treat other pathologies affecting the central nervous system, repair processes in cardiac tissue, and angiogenesis induction for ischemic diseases (10,13,23).

The present study was designed to evaluate the effect of ASC transplantation in mice suffering from chronic liver injury. Recipient mice were exposed to repeated CCl₄ administration to induce chronic liver injury, and the experiments were conducted at a stage of early fibrosis. To establish autologous cell-based therapy for chronic liver injury, ASCs were isolated from mice from the same genetic background as the recipient mice. A single

transplantation procedure using ASCs was performed, and the safety profile and therapeutic efficacy were assessed to determine the medicinal value of this methodology.

MATERIALS AND METHODS

Animals

C57BL/6 mice (5 weeks old) were purchased from CLEA Japan (Tokyo, Japan). All animal studies were performed in accordance with the institutional guidelines set forth by the Animal Care Committee of Tokyo Women's Medical University. Mice were housed in cages within a temperature-controlled room with a 12-h light/12-h dark cycle as well as ad libitum access to food and water.

Isolation and Culture of Adipose-Derived Stem Cells (ASCs)

ASCs were obtained as described by Mazo et al. (11) with some modifications. ASCs were isolated from the inguinal adipose tissues of 10-week-old male C57BL/6 mice. Inguinal adipose tissues were harvested and washed extensively with phosphate-buffered saline (PBS) to remove blood cells. The adipose tissues were cut finely and digested with collagenase A (2 mg/ml, Roche, Barcelona, Spain) at 37°C for 30 min. After filtration (100- μ m filters), the cells were centrifuged at 600 $\times$ g for 10 min at room temperature. The cells were washed three times in PBS. The pellet was resuspended in culture medium [DMEM/F12 (Invitrogen, Carlsbad, CA) with 10% FBS (JRH Bioscience, Kansas, USA), 100 IU penicillin, and 100 mg/ml streptomycin (Invitrogen)]. The cells were plated at 6×10^4 cells/cm² on 75-cm² tissue culture flask (Sumitomo Bakelite, Tokyo, Japan) and incubated at 37°C/5% CO₂. The primary cells were cultured until they grew to 80–90% confluency and passaged. In this study, ASCs at passage 3 were used for in vitro and transplantation experiments.

Pluripotency of ASCs

Osteogenic Differentiation. ASCs were seeded at 1×10^4 cells/cm² and cultured overnight. The culture medium was then switched to promote osteogenesis by adding 100 nmol/L dexamethasone, 10 mmol/L β -glycerolphosphate, and 0.05 mmol/L ascorbic acid-2-phosphate (all biochemical grade chemicals from Wako Pure Chemicals, Osaka, Japan). The medium was changed twice a week.

After 3 weeks of culture, the osteogenic differentiation status of ASCs was confirmed by Alizarin Red S staining as previously described (4,9).

Adipogenic Differentiation. ASCs were seeded at 3×10^4 cells/cm² on 12-well tissue culture plate (Sumitomo Bakelite), and culture was continued until they reached confluency. The culture medium was then switched to promote adipogenesis by supplementing with 10 μ mol/L insulin, 0.5 mmol/L isobutylmethylxanthine, 1 μ mol/L dexamethasone, and 200 μ mol/L indomethacin (Wako Pure Chemicals). The medium was changed twice a week. After 10 days of culture, the adipogenic differentiation status of ASCs was confirmed by Oil Red O staining as described previously (4,9).

Induction of Chronic Liver Injury in Mice and ASC Transplantation

Chronic liver injury in C57BL/6 mice was induced by repeated administration of carbon tetrachloride (CCl₄). In brief, C57BL/6 male mice were injected subcutaneously with 4 ml/kg body weight of one-fourth diluted CCl₄ (Wako Pure Chemicals) with olive oil (Wako Pure Chemicals) twice a week for 4 weeks. One day after the eighth administration, the cultured ASCs were transplanted into the tail vein using a 27-gauge needle. The mice were divided into the following four groups: (1) no-ASC group: PBS (200 μ l) (sham control), (2) ASC-low group: 1.5×10^3 ASCs suspended in 200 μ l of PBS, (3) ASC-middle group: 1.5×10^4 ASCs suspended in 200 μ l of PBS, and (4) ASC-high group: 1.5×10^5 ASCs suspended in 200 μ l of PBS ($n=3$, in all the groups). After the ASC transplantation, CCl₄ was injected two more times to maintain chronic liver injury. One day after the final (10th) CCl₄ injection, mice were euthanized and blood and liver were taken for biochemical and histological analysis. The experimental design was illustrated in Fig. 1.

Analysis of Hepatic Marker Enzymes and Coagulation Activity in the Blood

Blood samples were collected in the presence of 0.1 volume of 3.8% sodium citrate and centrifuged at 3,000 rpm for 15 min at 4°C to obtain plasma. Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST),

Figure 1. Schematic illustration of experimental protocol.

Figure 2. Transplantation of ASCs into the mice with chronic liver injury. Morphology and bipotency of the adipose-derived stem cells (ASCs) used in this experiment. (A) Morphology of ASCs immediately prior to harvesting for transplantation. ASCs were grown in control condition using DMEM/F12 with 10% fetal bovine serum (FBS). (B) Oil Red O staining of the ASCs after adipogenic differentiation. (C) Alizarin Red S staining of the ASCs after osteogenic differentiation. Scale bars: 100 μ m.

lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) were analyzed using OLYMPUS AU 400 chemistry analyzer (OLYMPUS, Tokyo, Japan). The prothrombin time was measured using Thrombocheck PT Plus (Sysmex, Kobe, Japan) by KC4 Delta Coagulometer (Trinity Biotech, Wicklow, Ireland).

Histological Investigation

Mouse livers were fixed with 10% buffered formalin and embedded with paraffin. Specimens were sliced into

5- μ m-thick sections and stained with hematoxylin and eosin (H&E). Sirius Red staining was performed by incubating the sections with Sirius Red solution (0.1% Direct Red 80 in saturated picric acid) to visualize collagen fibers.

Statistical Analysis

Values were expressed as mean $\pm$ SD. Statistical difference was tested using one-way ANOVA and the Tukey's HSD test as a post hoc analysis using StatView software (SAS Institute, Cary, NC).

Figure 3. Biochemical analyses for assessing liver injury status and hepatic functions. One week after ASC transplantation in carbon tetrachloride (CCl₄)-treated or nontreated mice, serum samples were analyzed for specific biochemical markers of liver and extrahepatic injury. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase. $n=3$ in each group. * $p<0.05$, compared with all the other groups by two-way ANOVA followed by the Tukey's HSD test.

Table 1. Prothrombin Time (PT) in Mice With Chronic Liver Injury Received Adipose-Derived Stem Cell (ASC) Transplantation

CCl ₄	ASCs	PT (s)
(-)	no	10.9±0.4*
(+)	no	13.4±3.2
(+)	low	13.5±1.1
(+)	middle	13.0±0.6
(+)	high	13.6±1.3

One week after the ASC transplantation to the carbon tetrachloride (CCl₄)-treated or nontreated mice, plasma samples were harvested for PT. Values were expressed as mean±SD. Low, 1.5×10³ ASCs transplanted; middle, 1.5×10⁴ ASCs transplanted; high, 1.5×10⁵ ASCs transplanted.

**p*<0.05, compared with all the other groups by two-way ANOVA followed by the Tukey's HSD test.

RESULTS

Morphological Determination of Bipotent Differentiation of ASCs to Adipocytes and Osteoblasts

Cells isolated from the adipose tissue were plated, and some cells were found to adhere to the plastic culture dish. These adherent cells progressively proliferate with the phenotypic morphology of fibroblasts (Fig. 2A). To initiate the adipogenic differentiation process, cells were maintained in adipogenic medium for 10 days, which altered the ASC morphology from fibroblast-like to a more spherical shape. Oil Red O staining showed numerous red-stained lipid droplets (Fig. 2B). ASCs were also cultured in osteogenic medium for 3 weeks. These cells formed osteoblast-like morphology and were positive for Alizarin Red S staining (Fig. 2C).

Figure 4. Representative photomicrographs of histological and histochemical analyses of the liver. One week after ASC transplantation in CCl₄-treated or nontreated mice, liver samples were harvested for histological analyses from non-CCl₄ group (A, B), no-ASC group (C, D), and ASC-high group (E, F). Sections were stained by H&E staining (A, C, and E), and Sirius Red staining (B, D, and F). Scale bars: 500 μ m.

Plasma Assessments for Liver Injuries and Liver Functions

No mouse died from CCl₄ intoxication regardless of whether the ASC transplantation procedure was performed. Serum ALT and AST levels of CCl₄-intoxicated mice showed significantly elevated values (4500±124 and 5200±140 IU/L, respectively) compared with the normal (control) mice, which exhibited low levels of ALT (46.4±5.5 IU/L) and AST (55.0±10.5 IU/L). No significant difference in serum ALT and AST was calculated between the three groups of ASC-treated mice (i.e., ASC-low, ASC-middle, and ASC-high) (Fig. 3A, B).

To assess if ASC transplantation was associated with any other biological side effects, LDH and ALP activities were determined (Fig. 3C, D). There were no statistical differences among the no-ASC, ASC-low, ASC-middle, and ASC-high groups. We also determined plasma prothrombin time, a sensitive marker of liver function, but no significant improvement was also detected among all CCl₄-intoxicated groups (Table 1).

Histological Assessments for Liver Injuries and Fibrosis

To determine whether ASC transplantation could beneficially attenuate CCl₄-induced liver injury from progression toward fibrosis, histological analysis was performed using liver samples obtained 1 week after transplantation. In the period between ASC transplantation and euthanization, the CCl₄ inoculation was continued twice a week. As shown in Figure 4, massive liver necrosis and hepatocyte vacuolization was found in all of the CCl₄-intoxicated groups. However, there were no observable histological changes in terms of liver necrosis and hepatocyte injuries between the groups with and without ASC treatment.

We also conducted Sirius Red staining using liver samples harvested 1 week after ASC transplantation. In all of the CCl₄ inoculated groups, the Sirius Red staining-positive fibrosis formation was localized within the pericentral area, confirming that repeated CCl₄ injections induced mild fibrosis (Fig. 4). Lobular disorganization was not observed in any of the CCl₄ administered groups. Again, no observable histological differences were found in terms of fibrosis between the groups with or without ASC treatment.

DISCUSSION

The present study analyzed the therapeutic safety of a single ASC transplant procedure in the context of chronic liver disease. Our results showed that ASC transplantation into mice in the early stages of chronic liver injury does not induce any adverse events in the liver. However, this approach did not significantly improve the liver injury state as determined by plasma parameters and histology in the treated versus control mice.

In view of the limited availability of allogenic cells for the application of regenerative medicine, mesenchymal stem cells, including ASCs, from an autologous tissue source has begun to receive considerable attention (13). In the present culture condition, ASCs have shown their ability to continuously renew through their progressive cell proliferation for four or more passages. The bipotency of the ASCs utilized in the present study was determined *in vitro* by inducing adipogenic and osteogenic differentiation. The differentiation potential of the ASCs was confirmed by morphological alterations as well as positive Oil Red O and Alizarin Red S staining for adipogenic and osteogenic differentiation, respectively.

In the present study, ASC transplantation was performed in CCl₄-intoxicated mice by injecting the cells into the systemic circulation via the tail vein. Our findings highlight the value of ASCs for a number of reasons: (1) a high yield recovery from autologous tissue, (2) the relatively easy maintenance of bipotent stem cells *in vitro*, and (3) safety in the process of transplantation. Another object of this study was to investigate therapeutic effects of ASCs on chronic liver failure after a short-term period after the transplantation procedure. No observable therapeutic effects were found regardless of the amount of ASCs transplanted 1 week after the transplantation of ASCs. Since there would be a possibility that the effects may be gained at a later phase after ASC transplantation, a prolonged period of observation may be necessary in the future study.

CCl₄ is a substantially toxic agent that induces broad liver necrosis at pericentral area of the liver (12,20). It is possible that the additional treatments with CCl₄ following the ASC transplantation resulted in a more severe and accelerated liver injury, such that any benefits associated with the ASCs were outweighed by the chemical damage.

There is a recent report in which ASC engraftment is less favorably located in the liver under nondisease conditions and more likely to be engrafted into the lung (28). In contrast, when cell infusion was performed to the mouse with severe liver fibrosis, highly efficient cell engraftment along the liver fiber was documented in studies utilizing ASCs and other cell types, including bone marrow cells or embryonic stem cells (5,14,22). The livers in these studies were severely fibrotic (5,14,22) compared with the minimally fibrotic livers in our study. Although it remains unclear as to why the liver fibrosis state supports ASC migration and engraftment, the environmental “niche” associated with the fibrotic state may be a plausible factor. In our mouse model, no improvement in liver injury was observed following ASC transplantation, which was consistent with recent studies by Goldenberg and others (2,21), who used bone marrow cells instead of ASCs. From these findings, there may be differences in the state of liver disease leading to differences in the

environmental “niche” and ultimately altering the effectiveness of cell engraftment within the liver.

To achieve higher ASC engraftment in livers undergoing chronic disease progression, it is likely important to establish optimal transplantation procedures, including cell infusion approaches and methods to precondition the recipient tissue. Several beneficial factors may be obtained from previous hepatocyte transplantation studies (17). Recently, Goto et al. (3) documented a unique cell delivery procedure to the liver in which these cells were transferred in a retrograde fashion via the hepatic vein allowing cells to predominantly engraft around the hepatic vein (zone 3). In the clinical setting, this retrograde cell infusion approach can be easily performed using a balloon catheter under fluorographic procedures (6). Other important factors that may contribute to enhance ASC engraftment may be to control the vascularity in the liver (25), reverse the disruption of the sinusoidal endothelium (7), inhibit the activation of the coagulation cascade (26,29), and/or combine tissue engineering technologies (15,16,18,19,27).

In conclusion, this is the first report to assess the feasibility of cell therapy using ASCs in a state of chronic liver injury. We described that a single application of ASC transplantation failed to provide any significant therapeutic improvement in mitigating the progression of the chronic liver disease. Given the fact that the therapeutic potential of ASCs has been reported in many diseases and disorders, additional refinements in the timing of delivery and the route of administration may need to be optimized to use these particular cells for treating chronic liver diseases.

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