ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELLS ATTENUATE FULMINANT HEPATITIS IN MICE INDUCED BY CONCANAVALIN A

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Abstract Stem cell therapy is one of the remarkable treatments for fatal diseases. Mesenchymal stem cells (MSCs) are adult stem cells and detected in various tissues such as bone marrow, umbilical cord blood and adipose tissue. MSCs have some abilities to differentiate into multi-lineage mature cells, modify immune responses and play a role for tissue repairing. The purpose of the present study was to investigate the effect of adipose tissue-derived mesenchymal stem cells (ASCs) against fulminant hepatitis, a life threatening liver injury due to massive destruction of hepatocytes.

BALB/c mice were sacrificed and adipose tissues were harvested. ASCs were purified from the tissues and confirmed by characterization of cell surface markers and induction of differentiation into multi-lineage mature cells. Concanavalin A (ConA), a protein of jackbean, can induce fulminant hepatitis for mice. BALB/c mice were administered lethal or sublethal dose of ConA intravenously and treated by ASCs, phosphate buffered saline (PBS) or splenocytes (SPLCs). The survival rates, liver enzymes, cytokines, histopathological changes and localization of ASCs were investigated.

ASCs could increase the survival rates, inhibit elevation of liver enzymes and cytokines, and attenuate necroses of hepatocytes compared to PBS or SPLCs. Fluorescent stained ASCs were detected in inflammatory liver, but not in normal liver.

These findings suggest that ASCs have an ability to improve or attenuate fulminant hepatitis.

Key words: acute liver failure; autoimmune hepatitis; mesenchymal stem cell; cell therapy

Introduction

Fulminant hepatitis is a severe state of liver injury because of massive destruction of hepatocytes caused by excessive immune responses, viral eradication, or chemical reactions. It progresses rapidly toward death if patients are not provided with a quick and appropriate treatment¹,². Although there are many medical treatments to recover liver function, liver transplantation is the only definitive way to improve the situation. However, there are many unsolved problems relating to liver transplantation such as lack of donors, operative damages, risk of rejection, and side effects of immunosuppressants³. Alternative effective treatments for fulminant hepatitis without relying on liver transplantation are expected.

Concanavalin A (ConA)-induced liver injury in mice is a well-known model of fulminant hepatitis due to excessive immune responses⁴,⁵. It is believed that T cells and NKT cells play a crucial role for establishment of ConA-induced hepatitis⁶, and cytokines including tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) produced by those cells are exacerbating factors.
Mesenchymal stem cells (MSCs) are one of the somatic stem cells residing in bone marrow and other various tissues. MSCs possess the abilities to differentiate into adipocytes, osteocytes, and chondrocytes to regulate immune system, and to play a role for tissue repairing. MSCs can particularly affect various immune cells for suppressing their activation and proliferation.

Previous studies have shown the immunoregulatory effects of MSCs on T cells and NKT cells. We therefore hypothesized that MSCs would be able to inhibit the activation of T cells and NKT cells in a mouse model of ConA-induced hepatitis leading to a reduction of liver injury and mortality.

MSCs also exist in adipose tissue, and they have the similar abilities of differentiation and immunoregulation as MSCs residing in bone marrow. They are easy to obtain from adipose tissue and to establish.

In the present study, the therapeutic efficacy of adipose tissue-derived mesenchymal stem cells (ASCs) for ConA-induced hepatitis was demonstrated.

**Materials and Methods**

**Mice**

BALB/c mice (Clea Japan, Tokyo, Japan), 8-12-week-old females, were housed in plastic cages with controlled light and dark cycle and fed a standard diet with water ad libitum. All the animal experiments were performed in accordance with Guidelines for Animal Experimentation of Hirosaki University.

**Isolation and expansion of ASCs**

We used a method for isolation and expansion of ASCs as previously described with minor modification. Subcutaneous and visceral adipose tissues were taken from BALB/c mice. Collected tissues were placed in Hank’s balanced salt solution (HBSS). Tissues were minced finely and incubated in 3 ml of Iscove’s modified Dulbecco’s media (IMDM, Invitrogen, NY, USA) containing 0.2% of collagenase (type II, Sigma, St. Louis, USA) and 1% bovine serum albumin (BSA, Sigma) at 37°C for 40 min. Cells were collected from digested tissue through 70-μm mesh. After centrifuging at 800 x g for 10 min and discarding supernatant, 3 ml of 0.83% NH₄Cl was added to hemolyze, and the cells were washed twice with HBSS and once with IMDM. Cells were seeded in IMDM containing 9% fetal bovine serum (FBS), 9% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The medium was changed every 3 or 4 days. Confluent cells were treated with 1 mM EDTA containing 0.25% trypsin for 2 min at 37°C and non-adherent cells were collected. Furthermore, the medium was changed every 3 or 4 days for 1 to 2 weeks. The cultured cells were used for the examinations.

**Differentiation of ASCs**

To ensure that the cultured cells were ASCs, an ability to differentiate to multi-lineage cells was investigated. For differentiation to osteocytes, adipocytes, and chondrocytes as mesodermal lineages, StemXVivo™ (R&D Systems, Minneapolis, USA) medium and supplements were used according to the manufacturer’s instructions. Two to 4 weeks later, each cell population was stained by Oil red O staining, von Kossa staining, and Alcian blue staining for adipocytes, osteocytes, and chondrocytes, respectively.

**Flow cytometric analysis for cell surface markers**

Cultured cells were detached from cultured flask by 0.25% trypsin with 1 mM EDTA for 2 min at 37°C and re-suspended in phosphate buffered saline (PBS) containing 2% FBS (2% FBS-PBS). The cells were counted and 1.0 x 10⁶ cells were collected by centrifugation at 800 x g.
for 5 min. After discarding supernatants, 1 μl of anti-CD16 plus anti-CD32 antibodies (Abcam, Cambridge, UK) was added and incubated at 4°C for 30 min. The cells were labeled with phycoerythrin (PE)-labeled anti-CD34 antibody (GeneTex, Irvine, CA, USA), PE-labeled anti-CD45 antibody (R&D Systems), PE-labeled anti-CD117 (c-kit) antibody (R&D Systems), PE-labeled anti-CD90.2 antibody (Birmingham, AL, USA), fluorescein isothiocyanate (FITC)-labeled anti-CD106 antibody (Funakoshi, Tokyo, Japan), and PE/Cy5.5-labeled anti-Sca-1 antibody (GenWay Biotech, San Diego, CA, USA). The suspensions were incubated at 4°C for 30 min and washed with 2% FBS-PBS. Then the cells were analyzed by FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

**Isolation of splenocytes**

Mice were sacrificed by cervical dislocation. The spleen was harvested and minced by scissors, and then the cells were filtered through 70-μm mesh. After centrifuging at 800 x g for 10 min and discarding supernatant, 3 ml of 0.83% NH₄Cl was added, and the cells were washed twice with RPMI1640 medium containing 10% FBS. The cells were then resuspended in 2% FBS-PBS, counted and prepared at the concentration of 2.0 x 10⁶ cells/ml before injection.

**Induction of ConA hepatitis and survival analysis**

Mice were injected intravenously with 30 to 50 mg/kg (body weight) of ConA (Sigma) diluted with PBS. Most of the mice injected with more than 40 mg/kg of ConA died within 72 h. In contrast, all of the mice administered with less than 35 mg/kg of ConA survived (data not shown). Based on this result, 40 mg/kg of ConA was used as the lethal dose in this study.

For survival analysis, mortality of mice was recorded every 3 h up to 72 h or until all mice died.

**Treatment**

ASCs and SPLCs were suspended in 2% FBS-PBS at the concentration of 2.0 x 10⁶ cells/ml. At 30 min after ConA administration, mice were injected intravenously with 1.0 x 10⁶ ASCs, 0.5 ml 2% FBS-PBS, or 1.0 x 10⁶ SPLC for ASC group, control group, or SPLC group, respectively.

**Measurement of liver enzymes and cytokines**

Mice were anesthetized by diethyl ether and whole blood was obtained by cardiac puncture using with 23G needles. Blood samples were centrifuged at 2300 x g for 30 min, and sera were collected.

For measurement of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, mice were injected with 20 mg/kg of ConA and treated as described previously. Twenty-four hours after hepatitis induction, sera were collected for AST and ALT measurement by SPOTCHEM EZ® (ARKLEY, Kyoto, Japan).

For cytokines analysis, mice were injected 40 mg/kg of ConA and treated. Sera were collected at 2 h, 6 h and 12 h after hepatitis induction, and IFN-γ and TNF-α were measured by enzyme-linked immunosorbent assays (ELISAs). Determination of IFN-γ was carried out as described previously[22]. TNF-α was measured by using CytoSet™ ELISA kits (Invitrogen) according to the manufacturer’s instructions.

**Histological analysis**

Mice were sacrificed by cervical dislocation at 24 h after injection of 20 mg/kg of ConA, and livers were harvested and fixed with 10% formaldehyde, and thereafter cut horizontally into two pieces. After embedding in paraffin, livers were sectioned at 5-μm in thickness and stained with hematoxylin and eosin. The pictures of whole livers were taken and all necrotic areas were painted in black and the percentages of necrotic areas were analyzed by ImageJ.
software.

Detection of localization of ASCs

Fluorescent staining was used to trace injected ASC in mice. ASCs were stained with NEO-STEM® (Biterials, Seoul, Korea) according to manufacturer's instructions. Mice were divided in two experimental groups; hepatitis group and control group. Hepatitis group was injected 20 mg/kg of ConA and control group was 0.2 ml of PBS. At 30 min after injection, each group was administered $1 \times 10^6$ ASCs stained with NEO-STEM®. Twenty-four hours after the first injection, mice were sacrificed and the lungs, the liver, the spleen and the mesenteric lymph nodes were harvested. Those organs were fixed with 10% formaldehyde and embedded in paraffin, and then sectioned at 5-μm in thickness to be observed under fluorescent microscope (IX71, OLYMPUS).

Statistics

Statistical analysis was performed using Student’s $t$-test. In case of a survival curve, the data were analyzed by the log-rank test. Significant difference between experimental and control group was considered by $p$ value of <0.05. All data were given as a mean ± standard deviations.

Results

Characteristics of isolated ASCs

Multi-lineage differentiation into adipocytes, osteocytes and chondrocytes were induced, and each population was stained by Oil red O stain, von Kossa stain and Alcian blue stain, respectively. Differentiated cells were detected by each respective staining procedure (data not shown). These results indicate that ASCs can differentiate into multi-lineage cells.

Cell surface markers were also detected from expanded cells by flow cytometry. ASCs were negative for CD34, CD45 and CD117 (c-kit) and positive for CD90.2, CD106 and Sca-1 (data not shown).

Survival rate was significantly improved in ASC-injected mice, but the effect was observed only in high dose and early administration group

The survival rate of ASC-injected mice was significantly improved compared to control group and SPLC group. There was no difference of survival rates between control group and SPLC group (Figure 1A).

Next, dose dependency of ASCs was investigated. Survival rates were not improved in the groups injected with $1 \times 10^5$ or $5 \times 10^5$ ASCs ($p = 0.45$ and 0.73, respectively), but were significantly improved in $1 \times 10^6$ ASC-injected mice (Figure 1B).

All the mice survived 72 h could recover from severe condition and live over a month.

Elevation of liver enzymes in sera was attenuated in ASC-injected mice

In order to evaluate the severity of liver injury by measuring liver enzymes, sera were harvested 24 h after ConA injection, and the level of AST and ALT was measured. In control and SPLC groups, AST and ALT was markedly elevated, but the level of these enzymes was significantly suppressed in ASC group (Figure 2A).

ASCs inhibited production of cytokines

Mice were injected with 40 mg/kg of ConA and treated as the same method described above. At 2 h, 6 h, and 12 h of hepatitis induction, sera were harvested and TNF-α and IFN-γ titers were measured by ELISAs. Levels of these cytokines in ASC group were significantly lower than those of control and SPLC groups at each time point. In contrast, there was a significant reduction of IFN-γ in SPLC group compared to control group only at 6 h after induction of
Liver injury was ameliorated microscopically in ASC-injected mice

At 24 h after hepatitis induction with 20 mg/kg ConA, histopathological changes of livers obtained from ASC, control, and SPLC group were observed microscopically. Necrotic areas were mainly observed in the peripheral areas of each hepatic lobe (data not shown).

Percentages of necrotic areas were analysed by ImageJ software. The necrotic area of livers from ASC group was significantly lower than that of control and SPLC group (data not shown).

Injected ASCs were detected in the liver of ConA injected mice

Under fluorescent microscopic observation, NEO-STEM®-labeled ASCs were detected in the lungs of hepatitis group or control group, and there was no significant difference between each group. However, significantly high amount of NEO-STEM®-labeled cells were observed in the livers of hepatitis group compared to control group. Fluorescent positive cells mostly existed around Glisson's sheaths (data not shown).

Discussion

ConA-induced hepatitis is well known as a murine model of fulminant hepatitis due to excess immune responses, such as autoimmune hepatitis. ASCs are noticed as a kind of cells showing immunoregulatory functions, and their therapeutic abilities for liver injury were recently reported. However, the relationship between ASCs and fulminant hepatitis by ConA has not been well elucidated. In the present study, we have investigated the effect of ASCs for ConA-induced hepatitis.

ASC treatment improved the survival rate in ConA-induced hepatitis compared to control group. In SPLC group, even after receiving

![Figure 1. The survival rate was improved by ASC treatment in ConA-induced hepatitis.](image)

Mice were injected with 40 mg/kg of ConA and survival rates were observed every 3 h until 72 h. A) ASC group (N=14) significantly survived compared to control (N=12), and SPLC (N=17) groups. B) 1 x 10⁶ ASC- (N=14) treated groups could archived long term survival compared to control group, 1 x 10⁵ ASC- (N=8), 5 x 10⁵ ASC- (N=9)**; p < 0.01 vs Control. Data are cumulative values of at least two independent experiments.
the same number of splenocytes as ASCs, the survival rate was not improved. This result suggests that an efficacy of ASCs on hepatitis is not due to the number of cells but some specific features of the ASCs. Furthermore, ASCs seem to act as a therapeutic effector by themselves, not a trigger for host's therapeutic reactions, since survival rates were not improved in groups with low doses of ASCs.

There was improvement from liver injury in ASC group as evidenced by survival rates, levels of serum liver enzymes and histopathology data. AST and ALT levels were supposed to be in tandem with small areas of necrotic hepatocytes detected in histopathology, and suppression of their appearance meant prevention of necrosis of hepatocytes and this correlated with improved survival.

Previous reports have shown that ConA-induced hepatitis is mainly due to activation of NKT cells and T cells, and these cells produce TNF-α and IFN-γ. Suppression of TNF-α and IFN-γ production in these cell populations might be due to attenuation of liver injury in ASC-injected mice, therefore involvement of ASCs and NKT cells or T cells interaction is speculated.

Mechanisms of the immunoregulatory effect of ASCs are reported to be due to direct cell-

Figure 2. The levels of serum AST and ALT were normalized and serum TNF-α and IFN-γ were suppressed by ASC treatment in ConA-induced hepatitis.

Mice were injected with 20 mg/kg or 40 mg/kg of ConA and treated by PBS or 1 x 10⁶ ASCs or 1 x 10⁶ SPLCs. A) Sera were harvested at 24 h after hepatitis induction. AST and ALT were significantly low in ASC treated group (***: p < 0.01, *: p < 0.05 vs control). Data are shown as mean of three independent experiments (N=15). B) Sera were harvested at 2, 6 and 12 h later. TNF-α and IFN-γ titers were significantly low in ASC treated group (***: p < 0.01, *: p < 0.05 vs Control). Data are shown as mean of two independent experiments (N=10).
to-cell contact and their products such as indoleamine 2,3-dioxygenase, prostaglandin E2 and nitric oxide. Furthermore, it has been reported that ASCs can induce differentiation and activation of regulatory T cells (Tregs). Since Tregs suppress immune response, it is possible that ASCs modulate innate immune responses through Treg expansion and lead to alleviation of liver injury induced by ConA. These are three possible mechanisms, namely a direct interaction by cell-to-cell contact, an indirect influence by products from ASCs, and an indirect effect by expanding Treg cells, in protection from ConA-induced hepatitis. In the present study, fluorescent stained ASCs were detected in inflammatory liver more than normal liver. These results suggest a possibility that ASCs migrate from systemic circulation into inflammatory lesion and suppress the activities of NKT and T cells stimulated by ConA. Therefore a mechanism including a direct interaction by cell-to-cell contact might be possible.

ASCs can be harvested easily and less invasively in humans, such as liposuction, indicating that ASC is an attractive source of stem cells for clinical use. However, safety of ASCs administration still remains controversial. First, intravenous injection of a large amount of ASCs induces pulmonary embolism especially in mice. Secondly, ASCs can be a vector of malignant cells and help their growing. Therefore, these problems should be solved before clinical use.

In conclusion, ASCs have various potentials for application as stem cell therapies. Additionally, ASCs harvesting procedure is easy and minimally invasive for humans. As our study demonstrated, ASC treatment for fulminant hepatitis is effective and has a potential to be an innovative therapy for severe hepatic failures, but the safety and its mechanisms have not been well elucidated. Further investigations are necessary.

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