Mesenchymal stem cell therapy on murine model of nonalcoholic steatohepatitis

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Mesenchymal stem cell therapy on murine model of non-alcoholic steatohepatitis.

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Running Head:

Mesenchymal Stem Cell Therapy on Liver Disease

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Abstract

A severely malfunctioning liver, due to acute liver injury or chronic liver disease, can lead to hepatic failure. The ultimate treatment for hepatic failure is liver transplantation; however, the availability of donors is a critical issue. Therefore, regenerative therapy is an anticipated novel approach for restoring liver function. Mesenchymal stem cells are pluripotent somatic cells that can differentiate into several cell types, including hepatocytes. Moreover, they are obtainable from easily accessible autologous adipose tissue, making them ideal for regenerative therapy. This chapter describes experimental methods for isolating mesenchymal stem cells from murine adipose tissues and expanding them, and also describes murine chronic liver disease, steatohepatitis, for the study of experimental regenerative treatments of chronic liver disease.

Introduction

Liver disease is a major health issue worldwide, and includes chronic hepatitis and acute liver failure due mostly to infection by hepatitis or other viruses and drug hepatotoxicity (1). The most intense form of acute liver injury is fulminant hepatitis, which results in rapid and massive destruction of hepatocytes, leading to acute hepatic failure. By contrast, the pathological features of chronic liver diseases are characterized by persistent hepatic inflammation and subsequent fibrotic change that distorts the fine lobular architecture of the liver tissue. This ultimately leads to end-stage chronic liver injury, which manifests clinically as encephalopathy, due to the failure of various metabolic processes and impaired portal circulation. The liver is unique in that hepatocytes *per se* (2) or progenitor cells (3, 4) can proliferate and restore the original architecture and function of the liver. However, with massive destruction of parenchymal hepatocytes or chronic distortion of the liver architecture with advanced fibrosis the liver cannot regenerate sufficiently. The most effective and radical treatment for hepatic failure is liver transplantation. However, this is limited by the availability of donors, as there are too few donors compared to the population of hepatic failure patients. Even when a donor is

available, the relatively high mortality of the transplantation procedure and the permanent requirement for immunosuppressants are major burdens to the recipient.

Regenerative therapy is a novel alternative treatment to liver transplantation for the severely impaired, malfunctioning cirrhotic liver. Bone-marrow stem cells are thought to contribute to liver regeneration (5-8), although it is controversial whether bone-marrow hematopoietic stem cells can differentiate into hepatocytes (9-12). Mesenchymal stem cells are pluripotent somatic stem cells that can differentiate into mesodermal lineage cells, such as adipocytes, chondrocytes, and osteocytes (13), as well as into non-mesodermal lineage cells, such as cardiomyocytes (14, 15) and hepatocytes (16-19). They reside in the bone marrow, umbilical cord, and adipose tissues; adipose tissues are especially rich in mesenchymal stem cells. For regenerative cell therapy, autologous cells would be ideal, avoiding the requirement for matching the major histocompatibility antigens to prevent immunological rejection. Consequently, bone marrow and adipose tissues are attractive sources of mesenchymal stem cells for regenerative therapy. Mesenchymal stem cells may also have favorable biological effects on fibrosis (20, 21) and inflammation (22). This chapter describes experimental methods for studying regenerative therapy for chronic liver disease using mesenchymal stem cells, the

culture of mesenchymal stem cells from murine adipose tissue, and a murine model of steatohepatitis that resembles human non-alcoholic steatohepatitis (23). Other methods and their application to liver disease models are also discussed.

2. Materials

2.1. Reagents

1. Collagenase type I (Wako Pure Chemical Industries, Osaka, Japan)
2. Phosphate-buffered saline without calcium, magnesium (PBS(–)) (Wako Pure Chemical Industries)
3. DMEM/nutrient mixture Ham F-12 (DMEM/F12) with L-glutamine, 15 mM HEPES (Invitrogen, Life Technologies, Carlsbad, CA)
4. Fetal bovine serum (Invitrogen)
5. Antibiotic/antimycotic (100×), liquid (Invitrogen)
6. 0.05% w/v trypsin – 0.53 mmol/L EDTA-4Na (Wako Pure Chemical Industries)
7. Pentobarbital sodium (64.8 mg/ml) (Schering-Plough Animal Health, Tokyo, Japan)
8. Atherogenic and high-fat diet (ATH + HF): 38.25% CRF-1 (standard chow,

Charles River Laboratories Japan, Yokohama, Japan), 60.0% cocoa butter, 1.25% cholesterol, 0.50% cholate (Oriental Yeast, Tokyo, Japan).

9. Ethanol
10. α-Cyanoacrylate adhesive

2.2. Reagent preparation

1. Collagenase solution: 1 g of collagenase type I powder is dissolved in 133 ml of PBS and stored at –80°C until use.
2. Culture medium: DMEM/F12 supplemented with antibiotic/antimycotic liquid and 10% heat-inactivated FBS and stored at 4°C.
3. Dilute pentobarbital with PBS(–) at 10-fold for anesthesia of mice.

2.3. Animal

1. C57Bl/6J mice (male, 8–10 weeks old, Charles River Laboratories, Yokohama, Japan)

2.4. Equipment

1. Operating scissors
2. Tweezers
3. Needle
4. Needle holder
5. 15-ml polypropylene conical tube (BD Falcon, Franklin Lakes, NJ)
6. 100-μm cell strainer (BD Falcon)
7. 6-cm culture dish (Nunc, Rockside, Denmark)
8. 5-0 silk thread (Niccho Industry, Tokyo, Japan)
9. 27 gauge needle with 1 ml syringe

3. Methods

3.1. Isolation and culture of murine mesenchymal stem cells from adipose tissue

1. All animal experiments should comply with national laws and institutional regulations.
2. Euthanize a C57Bl/6J mouse by cervical dislocation.
3. Disinfect the skin with 70% ethanol.
4. Make a midline abdominal skin incision and peel off the skin to expose the subcu-

taneous inguinal region.

5. Obtain adipose tissue from the subcutaneous inguinal region by cutting the connective tissues between the adipose tissue and skin, and place in a 6-cm culture dish with PBS(–).
6. Remove the lymph nodes from the adipose tissue using tweezers (**See Note 1**).
7. Cut the obtained adipose tissue into 1~2-mm pieces with scissors.
8. Put the fragmented adipose tissue in a 15-ml conical tube containing 10 ml of phosphate-buffered saline.
9. Centrifuge it at 1,200 rpm for 3 min and remove the supernatant.
10. Add 10 ml of PBS (–) to the tube and centrifuge it at 1,200 rpm for 3 min.
11. Remove the supernatant as in step 8.
12. Put the PBS(–)-rinsed adipose tissue fragments into a 15-ml conical tube with 2~3 ml of collagenase aliquot.
13. Incubate the adipose tissue fragments and collagenase with shaking at 37°C in thermostat bath for 1 hour.
14. Add an equal volume of DMEM/F12 containing 10% heat-inactivated fetal bovine serum supplemented with 1% antibiotic/antimycotic liquid.

15. Centrifuge at 1,200 rpm for 10 min.

16. Remove the debris and PBS(–).

17. Resuspend the remaining cells in PBS(–) and filter them through a 100-μm cell strainer.

18. Centrifuge at 1,200 rpm for 10 min.

19. Remove the PBS(–), suspend the cells in 4 ml of DMEM-F12 supplemented with heat-inactivated FBS, and place in a 6-cm culture dish (Fig. 1a).

20. Replenish the culture medium with fresh complete medium the next day.

21. Replenish the medium every 3-4 days. The culture usually reaches 70% cell confluence after 10 days (Fig. 1a).

22. Cells can usually be passaged and expanded eight or nine times until morphological change appears (Fig. 1b) (**see Notes 2, 3**)

3.2. Establishing a murine steatohepatitis model

C57Bl/6J male mice are maintained in colony cages with a 12-hour light/12-hour dark cycle. Eight-week-old mice are fed an ATH + HF diet for 24 weeks. The livers of

these mice develop steatosis in hepatocytes accompanied with pericellular fibrosis (Fig. 2a, b), resembling the liver histology seen in advanced non-alcoholic steatohepatitis (23).

3.3. Experimental therapeutic application of mesenchymal stem cells in the murine steatohepatitis model

1. Mice that develop steatohepatitis on the ATH + HF diet for 24 weeks are anesthetized by an intraperitoneal injection of 200 μl of diluted pentobarbital.
2. Expanded mesenchymal stem cells isolated from murine adipose tissues are prepared.
3. A mid-abdominal incision is made and the middle lobe of the liver is exposed.
4. A 2~3-mm liver specimen is obtained by cutting with scissors and the cut area is closed using α-cyanoacrylate adhesive.
5. After the biopsy, a 1×10^5/200 μl mesenchymal stem cell aliquot is injected into the subcapsule of the spleen using a 27G gauge needle with a 1-ml syringe.
6. The peritoneum and skin are sutured with 5-0 silk.
7. The mice are kept on the ATH + HF diet for two more weeks.
8. After two weeks, the mice are euthanized by cervical dislocation. Serum and liver tissues are collected, and RNA is extracted from the liver tissues. These samples are

assayed to assess the therapeutic effect of administering mesenchymal stem cells **(see Notes 4, 5)**.

4. Notes

1. This step is required to avoid contamination of mesenchymal stem cell culture by resident lymphocytes.
2. The method of isolating and culturing mesenchymal stem cells from adipose tissues is described. Mesenchymal stem cells also reside in bone marrow, and the methods for isolating and expanding mesenchymal stem cells from bone marrow tissues have been reported (24, 25). The latter report states that mouse mesenchymal stem cells were isolated by aspiration of bone marrow in the tibia and femur, and cultured in DMEM supplemented with 15% FBS. The culture medium was replenished frequently. With this method, confluent mesenchymal stem cells can be obtained after 21 days.
3. CD105 is a marker for mesenchymal stem cells. Using the CD105 MultiSort Kit (Miltenyi, Auburn, CA), the mesenchymal cell fraction can be enriched.
4. To assess the effect of mesenchymal stem cells on the liver in the steatohepatitis

murine model, real-time quantitative PCR expression analysis of liver RNA samples was performed. Compared to the pretreatment level, expression of interleukin-6 was upregulated and that of interleukin 15 receptor alpha was downregulated after the mesenchymal stem cell treatment (unpublished observation).

5. The carbon tetrachloride (CCl_4)-induced chronic liver disease model is another model of chronic liver disease (5, 26) that can be used for experimental regenerative therapy. To establish this murine model, C57Bl/6 mice are intraperitoneally injected with 1 mL/Kg of CCl_4 for 4 weeks. These mice develop advanced fibrotic changes in the liver, *i.e.*, cirrhosis. The therapeutic effect of mesenchymal stem cells on chronic liver disease can also be studied using this model. It is reported that entire fractions of bone marrow cells can improve liver fibrosis in this CCl_4-induced cirrhotic murine model, presumably via the activation of matrix metalloproteinase (5). The rat is an alternative rodent for establishing chronic liver injury models, either CCl_4-induced (27, 28) or steatohepatitis (29) cirrhosis.

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Figure legends:

Figure.1

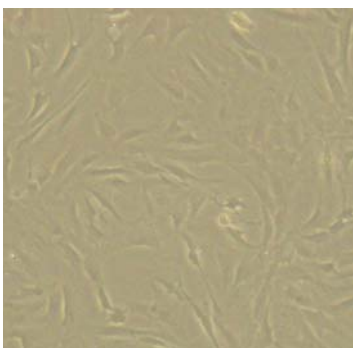
The appearance of cultured cells obtained and expanded from murine adipose tissues. (a) The characteristic “spindle shape” of the mesenchymal stem cells is observed. (b) Morphological change of cells appeared usually after 10 times passages. (c) CD105 expression of cultured cells (8 passages).

Figure 2.

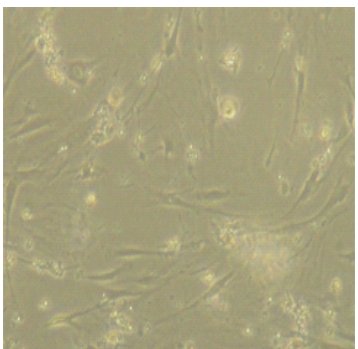
Histology of the liver obtained from mice which was fed with ATH+HF diet for 24 weeks. (a) HE staining (x100) (b) AZAN staining (x100)

Figure. 1

(a)



(b)



(c)

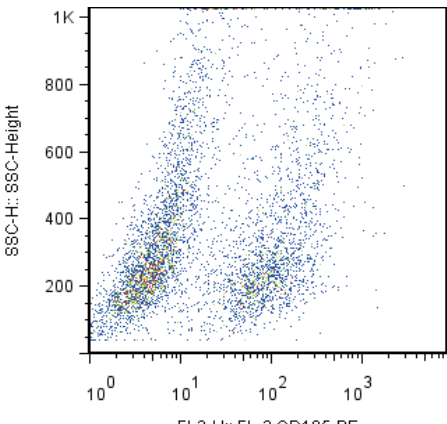
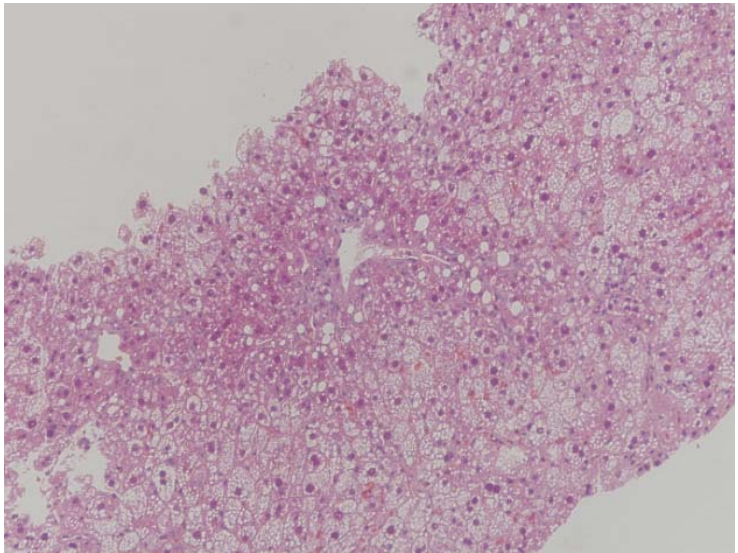

1K
800
600
400
200
SSC-H:: SSC-Height
10^0
10^1
10^2
10^3
FL2-H:: FL-2 CD105-PE

Figure. 2

(a)



(b)