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Therapeutic effect of transplanting HGF-treated bone marrow mesenchymal cells into CCl_4-injured rats

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Background/Aims: The autologous transplantation of bone marrow cells is a promising treatment for liver disease. Pluripotent bone marrow stem cells can differentiate into hepatocytes, but few reports address the therapeutic effect of transplanting these stem cells into damaged liver in vivo. Here, we transplanted bone marrow-derived mesenchymal cells (BMMCs) to test their effect in liver-injured rats.

Methods: Rat bone marrow cells were cultivated for 2 weeks in the presence or absence of hepatocyte growth factor (HGF), labeled with a fluorescent marker, and transplanted by injection into CCl_4-injured rats. Blood samples collected 4 weeks later were analyzed for albumin production and transaminase levels. The amount of fibrosis was determined by histology.

Results: RT-PCR analysis detected α-fetoprotein and albumin mRNAs in BMMCs cultured with HGF for 2 weeks. Albumin protein was also produced in the BMMC cultures by a subpopulation of cells. Transplantation of the BMMCs into liver-injured rats restored their serum albumin level and significantly suppressed transaminase activity and liver fibrosis. These effects were not seen when the BMMCs were cultured without HGF.

Conclusions: The transplantation of BMMCs cultured with HGF effectively treats liver injury in rats. This is a promising technique for autologous transplantation in humans with liver injury.
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Keywords: Bone marrow cells; Mesenchymal stem cells; Cell therapy; Liver injury; Autologous transplantation; Liver fibrosis

1. Introduction

Embryonic stem (ES) cells can be propagated indefinitely in the undifferentiated state, and the cells retain the ability to differentiate into all cell types. Despite their versatility, the use of ES cells in regenerative medicine faces several difficulties, including ethical issues and clinical complications such as immune reactions and teratoma formation. Bone marrow cells are presently considered to be the most promising cell source for the cell therapy of various diseases. Donor bone marrow cells transplanted into animal models of various diseases have been shown to differentiate into various cell types, including bone, cartilage, cardiac muscle, vascular endothelial, and neuronal cells [1–5].

Pluripotent hematopoietic stem cells (HSCs) contained in bone marrow cells can differentiate into hepatocyte-like cells in vivo [6,7]. Miyazaki et al. showed that hepatocytes were induced in vitro from bone marrow cells expressing an HSC marker that were grown in medium containing hepatocyte growth factor (HGF) [8]. Recently, Schwartz et al. reported that mesenchymal stem cells (MSCs) can differentiate into functional hepatocyte-like cells in vitro [9]. The addition of HGF and fibroblast growth factor-4 to the culture medium induces the differentiation. HGF was

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identified as a mitogen for mature hepatocytes [10–12], and is considered to be important for the development and regeneration of the liver [13]. Since, MSCs can be expanded in culture to retain their multilineage potential [1,14], the use of patients' own MSCs to treat liver disease is considered a promising strategy. MSCs are known to exist in bone marrow mesenchymal cells (BMMCs), which can adhere to culture substrate and proliferate while retaining the ability to differentiate into several cell types. Here, we propagated rat BMMCs on tissue culture plates and used them as our source of MSCs. HGF was used to direct the MSCs to differentiate in the hepatocyte lineage. The transplantation of BMMCs grown in the presence of HGF into CCl_4-injured rats was an effective treatment for their liver injury.

2. Materials and methods

2.1. Cell isolation and culture

Bone marrow cells were isolated from Fisher 344 rat femurs by flushing the femurs with α-MEM containing 15% fetal bovine serum (FBS, JRH Bioscience, Lenexa, KS, USA), 50 U/ml penicillin G, 125 ng/ml amphotericin B, and 50 μg/ml streptomycin, using a 21-gauge needle. Isolated bone marrow cells were seeded onto collagen-coated polystyrene plates at a density of 2×10^4 cells/cm^2, and cultured with Dulbecco's modified Eagle medium (GIBCO, Grand Island, NY, USA) containing 10% FBS, 100 mg/l insulin, 0.067 mg/l sodium selenium, and 55 mg/l transferrin (Medium A). Twenty nanograms per millilitre HGF (Genzyme TECHNE, Minneapolis, MN, USA) and 10 nM dexamethasone (Sigma-Aldrich, St Louis, MO, USA) were added to the Medium A in the priming culture to induce differentiation into hepatocytes. As a control, bone marrow cells were also cultured without HGF. Seven to nine days later, the majority of the cultured cells were adherent fibroblastic cells, as described later, negative for hematopoietic markers, and positive for known cell-surface antigens of mesenchymal cells. These fibroblastic cells can differentiate into bone/cartilage, endothelial cells, and cardiomyocytes [15–17]. During the cell culture with or without HGF, each medium change eliminated the floating cells, which were presumably hematopoietic cells, and allowed the adherent cells to proliferate. It is these adherent cells that we refer to as BMMCs in the present study. Hepatocytes were isolated from Fisher 344 rats by perfusing the liver with collagenase (from Clostridium histolyticum Type IV; Sigma-Aldrich) according to the method of Seglen [18]. The basal medium for hepatocyte culture (Medium B) consisted of 100 U/ml penicillin G, 100 μg/ml streptomycin, 50 ng/ml amphotericin B, and 100 ng/ml aprotinin (Nacalai Tesque, Kyoto, Japan) in William's medium E (WE, ICN Biochemicals, Costa Mesa, CA, USA). Medium C contained 10% FBS, 1 nM insulin (Sigma-Aldrich) and 1 nM dexamethasone (Nacalai Tesque, Kyoto, Japan) in the Medium B. Isolated hepatocytes were cultured with the Medium C for the first 6 h. The medium was changed to the Medium B, and hepatocytes were cultured in the absence or presence of CCl_4 for 18 h. BMMCs cultured for 2 weeks in the presence or absence of HGF were detached by trypsin treatment from the plate, and inoculated onto the hepatocyte layer. The BMMCs and hepatocytes were cocultured for 24 h with the Medium B.

2.2. RT-PCR

Total RNA was extracted from cultured cells using Isogen (Nippon Gene, Co., Ltd, Tokyo, Japan). The gene expression of albumin was analyzed using the following primers: forward 5′-CTTCAAAGCCTGGG-CAGTAG-3′ and reverse 5′-GCACTGGCTTATCACAGCAA-3′. The gene expression of α-fetoprotein (AFP) was analyzed using the following primers: forward 5′-CAGTGAGGAGAAACGGTCGG-3′ and reverse 5′-ATGGTCTGTAGGGCTCGGCC-3′. β-Actin gene expression was analyzed using the following primers: forward 5′-CATCCCC-CAAAGTTCTAC-3′ and reverse 5′-CCAAAGCCTTCATACATC-3′. RT was performed using a 1-μg total RNA sample with the RNA PCR kit AMV ver.3.0 (TaKaRa, Kyoto, Japan). The PCR conditions were: 1) 94 °C for 2 min; 2) 40 cycles of 30 s at 94 °C, 30 s at 50 °C for albumin, 56.2 °C for AFP, 56 °C for β-actin, and 1 min at 72 °C.

2.3. Immunocytochemical staining and western blotting of albumin

Cells were cultured in collagen-coated 12-well plates (Nippon Becton Dickinson, Tokyo, Japan) and fixed with 10% formaldehyde (WAKO Pure Chemical Co., Osaka Japan) for 20 min. Cells were treated with 0.5% Triton X-100 for several minutes, and then with blocking solution (Block Ace, Dainippon Pharmaceutical Co., Ltd, Osaka, Japan) for 1 h. The cells were incubated with primary antibody, i.e. rabbit IgG anti-rat albumin (Cappel, Durham, NC, USA) diluted 1:500 with the blocking solution, for 1 h. The cells were mixed with the secondary antibody, i.e. goat F(ab) anti-rabbit IgG (H&L)-Fluorescein (Leinco Technologies Inc., St Louis, MO, USA) diluted 1:500 with the blocking solution and incubated for 2 h. Between each step, the cells were washed three times for 5 min each with phosphate-buffered saline (PBS). All steps were carried out at room temperature.

Cytosolic fractions were prepared from detached cells for western blotting analysis using mammalian protein extraction reagent (M-PER, Pierce Biotechnology, Rockford, IL, USA). Cell lysates were normalized for protein concentration, subjected to one-dimensional sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 5–20% gradient), and proteins were electrophoretically transferred to PVDF membrane in a Bio-Rad Trans-Blot cell (140 mA, 90 min). Membranes were incubated in the blocking solution overnight at 4 °C, and then incubated with primary antibody, i.e. rabbit IgG anti-rat albumin (Cappel) diluted 1:5000 with PBS containing 0.1% Tween20 and 10% Block Ace for 1 h. Bound antibody was detected by using EnVision™ system kit (DakoCytomation, Glostrup, Denmark) containing horseradish peroxidase-conjugated anti-rabbit immunoglobulins as the secondary antibody and 3,3′-diaminobenzidine (DAB) as the substrate of color development. All steps except blocking were carried out at room temperature.

2.4. Transplantation experiments

BMMCs were cultured in the presence or absence of HGF for 2 weeks. After being detached from the plate by trypsin treatment, the cells were stained using the PKH Fluorescent Cell Linker Kit (Sigma-Aldrich). Fisher 344 rats aged 4 weeks were treated with 0.5 mg/kg CCl_4 by intraperitoneal injection, and 3×10^6 PKH-stained BMMCs were transplanted by injection into the caudal vein. After the transplantation, the rats were treated with 0.5 mg/kg CCl_4 twice a week for 4 weeks. Animal experiments were carried out in compliance with Japanese Law (No. 105) on animal protection and administration as well as the Regulation on the Implementation of Animal Experimentation of the AIST (Independent Administrative Organization, National Institute of Advanced Industrial Science and Technology).

2.5. Histopathologic staining

The liver was excised and immersed in hexane chilled with dry ice. Cryostat sections of 5 μm were cut and mounted on glass slides. PKH26-derived fluorescence was observed using a Typhoon 8600 variable mode imager (Amersham Biosciences, Piscataway, NJ, USA), and quantification of the intensity was carried out with Image Quant 5.1 software (Molecular Dynamics, Sunnyvale, CA, USA).

The liver specimens were fixed with 10% formaldehyde and embedded in paraffin. Tissue sections were mounted on slides and Azan staining was performed to analyze the extent of fibrosis. After establishing a color threshold for each micrograph, the number of pixels showing the blue color of the stained collagen fibers was extracted, and the percentage of these fibers in the liver was calculated by dividing the total blue-colored area by the total area of the liver.

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