ELSEVIER

Marrow-Derived Mesenchymal Stem Cells Restore Biochemical Markers of Acute Liver Injury in Experimental Model

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ABSTRACT

Bone marrow–derived mesenchymal stem cells were investigated as prompters of liver regeneration in an experimental model of acute hepatic injury. A model was created in Wistar rats through intraperitoneal injection of carbon tetrachloride (CCl4). Bone marrow–derived mesenchymal stem cells collected from the long bones of 10 Wistar rats were intravenously infused 24 hours after induction of acute liver failure in 16 rats, group A. In group B, the control group, 16 rats received a peritoneal injection of CCl4, and an intravenous infusion of normal saline solution. All rats were sacrificed at 2, 3, 4, and 7 days post-CCl4 injection to examined biochemical markers and pathological appearances. The platelet counts were higher in group A versus group B on post-CCl4 infusion days 2 ($P = .02$) and 3 ($P = .001$), as were the transaminase trends in glutamic oxaloacetic ($P = .002$), and glutamic-pyruvic transaminases ($P < .0001$). Pathological examination showed a greater grade of hepatocellular necrosis with neutrophilic infiltration in group B ($P = .02$). In conclusion, infusion of bone marrow–derived mesenchymal stem cell resulted in a less aggressive picture of hepatic damage.

LIVER DISEASES are among the first 10 causes of death worldwide, but treatment is hindered by a lack of effective pharmacological therapies. In fact, with the exception of antiviral drugs effective for hepatitis, there are few pharmacological means, to cure the acutely or chronically damaged liver.[1] Preclinical models of acute hepatic injury (AHI) offer the opportunity to investigate the pathophysiology of liver diseases and to test treatments. Experimental models with bone marrow–derived mesenchymal stem cells (MSCs) have led to developments in regenerative medicine.[2,3]

Liver transplantation is the most effective treatment for patients with hepatic failure, though it is dramatically limited by the shortage of available organs. Preclinical and clinical studies have investigated alternative therapies such as hepatocyte transplantation.[4,5] Adult hepatocytes, however, show limited proliferative potential, which is probably insufficient to repopulate a host liver.[6,7] Research studies using hematopoietic and bone marrow–derived MSCs have demonstrated their potentially multipotent regenerative pathway.[8]

Biochemical markers can be detected in the urine, blood, or other body fluids or tissues, serving as a sign of a disease or a functional abnormality. Normally, liver function is evaluated with certain biochemical markers, such as platelet count, international normalized ratio (INR), acute phase protein (ie, fibrinogen), transaminases (i.e., glutamate-pyruvate transaminase [GPT] and glutamate-oxaloacetate transaminase [GOT]), or alkaline phosphatase (ALP). The aim of this preclinical study was to investigate the role of MSC infusion to promote hepatic regeneration in the setting of AHI.

MATERIALS AND METHODS

Isolation and Purification of Bone Marrow–Derived MSCs

MSCs were collected by flushing the long bones of 10 Wistar rats (Harlan Nossan, San Pietro al Natisone, Udine, Italy) with Hanks' balanced salt solution (Gibco, Invitrogen, Milano, Italy). After

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0041-1345/13/$–see front matter
http://dx.doi.org/10.1016/j.transproceed.2012.06.087

approval by appropriate Internal Review Boards, all animal procedures were approved by national authorities, and conducted under anesthesia with 100 mg/kg of zoletil (Zolazepam Tiletamina, VIRBAC Srl, Milano, Italy).

Cells plated in 175-cm^2 flasks were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; without pyruvate; Gibco, Invitrogen, Milano, Italy) plus 20% fetal bovine serum (FBS) (Gibco, Invitrogen, Milano, Italy), 1% penicillin/streptomycin, and 1% glutamine. After 48 or 72 hours, the culture medium was changed to remove nonadherent cells. Adherent cells detached with 2 mmol/L of ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) without Ca^{++} and Mg^{++} were retrieved in new flasks 2 weeks after starting the culture, or each time cell confluence reached 80% to 90%. Culture growth and expansion of MSCs were fostered by the use of a MesenCult Proliferation Kit (Stem Cell Technologies, Milano, Italy) containing culture medium (MesenCult Basal Medium, Stem Cell Technologies, Milano, Italy) and a standardized, serum-containing supplement for rat mesenchymal stem cells (MesenCult Mesenchymal Stem Cell Stimulatory Supplements, Stem Cell Technologies, Milano, Italy).

Cytofluorimetric Assay

Cytofluorimetric characterization (FACS Aria II, Becton, Dickinson and Company, NJ, USA) of the heterogeneous bone marrow cell population was performed at the time of extraction from the bones to observe variations in the percentages of cultured MSCs. Upon extraction, the percentage of MSCs was approximately 0.001%, increasing with progression of the primary cell cultures. We compared the peripheral blood, the freshy extracted bone marrow and 10-day primary culture cells.

Cytofluorimetric characterization of MSCs examined surface antigens: $CD105^+$ (Santa Cruz Biotechnology); $CD45^-$, $CD34^-$; $CD14^-$, $CD90^+$, $CD73^+$, $CD29^+$ and $CD11b^+$ using FACSAria II cytofluorimetry/cell sorter and FACS Diva version 6.1.2 software (Becton, Dickinson and Company, NJ, USA). Living (7AAD-negative) cells were analyzed with Cell Quest version 6.0.2 software (San Jose, Calif, USA). Adherent cells treated with a 2 mmol/L solution of EDTA in PBS without Ca^{++} and Mg^{++} for 2 minutes at room temperature, were washed with medium for 5 minutes at 1500 rpm 20°C. The cell pellet was resuspended in sterile PBS. Staining of the cell suspension with each antibody was performed with 10 μL of for 30 minutes at room temperature with no exposure to light. Cells centrifuged in sterile PBS for 5 minutes at 1200 rpm yielded a pellet that was resuspended in sterile PBS for analysis by cytofluorimetry.[9]

Characterization of MSCs by Colony-Forming Unit Capacity Test

Differentiation of MSCs into adipocytes was initiated by plating them into 24-well plates in expansion medium without FBS for 2 days. After confluence had been reached in the expansion culture, they were cultured in insulin-supplemented expansion medium for 3 days without FBS (15 U/mL, Sanofi-Aventis, Milano, Italy), dexamethasone (10 - 6 mol/L, Sigma-Aldrich, Milano, Italy), goat serum (5 mL/100 mL, PromoCell, Heidelberg, Germany) and 3-isobutyl-1-methylxanthine (0.1 mg/mL, Sigma-Aldrich, Milano, Italy). For maturation, cells were then cultured in insulin-supplemented expansion medium without FBS (15 U/ml, Sanofi-Aventis, Milano, Italy) for 5 days.

Adipocytic differentiation was determined by Oil Red staining (Sigma, Milano, Italy) and hematoxylin counterstaining. Cells washed in cold PBS were fixed with 10% formaldehyde at 4°C overnight before incubation with 5 mg/mL Oil Red solution for 2 hours at room temperature.

Chondrogenic differentiation was achieved by pelleting 200,000 MSCs in a 15-mL polypropylene tube (1200 rpm, 5 minutes, 4°C) before 16-day treatment with chondrogenic medium consisting of high-glucose DMEM supplemented with 0.1 μmol/L dexamethasone, 0.2 μmol/L ascorbic acid, 0.2 μmol/L sodium pyruvate, 10 ng/mL transforming growth factor-β1, and 1% insulin, transferrin, selenium premix (Becton, Dickinson and Company, NJ, USA). After 16 days, pellets embedded in Tissue-Tek TEC 5 (Sakura Finetek USA, Inc, Calif, USA) were snap frozen. Chondrogenic differentiation was evidenced by Alcian Blue staining (Bio-Optica, Milano, Italy).

Osteogenic differentiation was induced by seeding MSCs in six-well plates at a density of 15000 cells per square centimeter in DMEM without FBS for 1 day before treatment with osteogenic medium for 2 weeks, changing the medium twice a week. Osteogenic medium consisted of DMEM supplemented with 0.1 μmol/L dexamethasone, 0.3 mmol/L ascorbic acid, and 10 mmol/L β-glycerol phosphate. Von Kossa staining was used to determine osteogenic differentiation. Cells were covered with 5% silver nitrate solution for 40 minutes in bright light, followed by an incubation step in ultraviolet light for 2 minutes, and incubation for 5 minutes in 1% pyrogallol, before rinsing again. Remaining silver nitrate was removed by washing the cells in 5% sodium thiosulfate for 5 minutes.

Recipient Groups

To establish the AHI rat model, 2 mL/kg CCl4 (1:1 dilution with olive oil) was injected into the peritoneal cavity of 36 adult Wistar rats of both genders (320 $\pm$ 20 g). The rats were divided into three groups: group A (n = 16) 24 hours later received an intravenous infusion of 3 million MSCs in 400 μL normal saline solution via the caudal artery using a 25-gauge needle; group B (n = 16), 24 hours later, an intravenous infusion of normal saline solution 0.9%; and group C (n = 4) only the CCl4 with sacrifice at 24 hours.

Four rats in groups A and B were sacrificed at 2, 3, 4, or 7 days respectively after the CCl4 injection.

Biochemical Markers for Evaluation of Liver Injury in the Serum

Blood samples were drawn in groups A and B on CCl4 postinfusion day 1 in group C, and postinfusion day 2 to postinfusion day 4, and postinfusion day 7. Liver function was assessed by platelet counts, INR, acute phase protein (ie, fibrinogen kinetic method), ALP, and transaminases (ie, GPT and GOT using UV-IFCC).

Liver Morphology

Biopsies performed at the time of the sacrifice were preserved in 10% buffered formalin and embedded in paraffin for routine pathologic examination. Liver histology was assessed by light microscopy (Olympus B X60, Tokyo, Japan) using hematoxylin and eosin–stained 5-mu sections. Two pathologists blindly assessed all biopsies. The extent of parenchymal damage was quantitatively estimated with the following criteria: the presence, extent, and location of hepatocellular necrosis and lobular neutrophilic infiltration; the presence and extent of hepatocellular ballooning; as well as micro- and macrovesicular steatosis.