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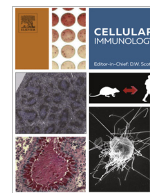

Research paper

Immunomodulatory effects of bone marrow mesenchymal stem cells overexpressing heme oxygenase-1: Protective effects on acute rejection following reduced-size liver transplantation in a rat model

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Here we explore the T-lymphocyte suppressive and immunomodulatory effects of bone marrow mesenchymal stem cells (BMMSCs) overexpressing heme oxygenase-1 (HO-1) on acute rejection following reduced-size liver transplantation (RLT) in a rat model. The proliferation activity, cell cycle progression, secretion of proinflammatory cytokines, expression of CD25 and CD71 in lymphocytes, and activity of NK cells were found to be significantly lowered, and the proportion of regulatory T cells (Tregs) was found to be increased relative to BMMSCs when Adv-HO-1/BMMSCs were co-cultured with Con A ex vivo; secretion of anti-inflammatory cytokines was significantly higher. When treated with saline, BMMSCs or Adv-HO-1/BMMSCs, post-transplantation rats receiving Adv-HO-1/BMMSCs showed better median survival time, lower rejection activity index, higher anti-inflammatory cytokine levels, lower proinflammatory cytokine levels, more peripheral Tregs, and lower natural killer cell viability. These results suggest that HO-1 enhanced and prolonged the effects of BMMSCs on acute rejection following RLT, with immunomodulatory effects in which adaptive and innate immunity, as well as paracrine signaling, may play important roles.

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1. Introduction

With improvement in surgical techniques and the use of new immunosuppressant drugs, long-term survival of transplanted organs has improved considerably, and liver transplantation has now become the most effective treatment for end-stage liver disease. However, shortage of donor organs and allograft rejection remain as major impediments to the development and success of liver transplantation. While successful implementation of split-liver transplantation can meet the shortage of liver to some extent, organ rejection after transplantation is still a problem that needs to be addressed urgently.

An immune response to transplanted antigen, mediated by the recipient's T cells, underlies allograft rejection and graft-versus-host reaction [1]. Recent studies have shown that bone marrow mesenchymal stem cells (BMMSCs) not only have pluripotency and self-renewal capacity, but also display immunomodulatory activity, which can have important applications in organ transplantation [2]. BMMSCs show low immunogenicity, and can suppress T cell-mediated rejection to some extent after organ transplantation [3]. BMMSCs exert immunosuppressive effects by secreting immunosuppressive cytokines, interacting with antigen-presenting cells (e.g., dendritic cells) [4–6], and interfering with Th lymphocyte differentiation. In addition, BMMSCs induce immune tolerance [7,8]

Abbreviations: ACR, acute cellular rejection; ANOVA, analysis of variance; BM MSCs, bone marrow mesenchymal stem cells; BN, Brown-Norway; Con A, concanavalin A; DMEM, Dulbecco's modified Eagle medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FCS, fetal calf serum; GFP, green fluorescence protein; H&E, hematoxylin and eosin; HO-1, heme oxygenase-1; LDH, lactate dehydrogenase; MHC, major histocompatibility complex; MSCs, mesenchymal stem cells; MTT, methylthiazolyl tetrazolium; NAD, nicotinamide adenine dinucleotide trihydrate; NK, natural killer; PI, propidium iodide; PMS, methylphenazinium methosulfate; POD, postoperative day; RAI, rejection activity index; RT-PCR, real-time polymerase chain reaction; SD, standard deviation; Tregs, regulatory T cells.

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and show reparative effects on ischemia–reperfusion injury [9]. These are properties that can be useful in clinical practice. However, research has demonstrated that BMMSCs after transfusion show low concentration and poor activity in the target tissues [10], moreover, their immune function needs to be improved if they are to have useful clinical impact [2]. The application of genetic engineering to alter BMMSCs gene expression has been proposed as a method for improving their effect [11].

Heme oxygenase-1 (HO-1) is an immunomodulatory factor involved in the regulation of immune tolerance after organ transplantation [12,13], and has also shown some ability for tissue repair [14]. HO-1 can be transduced into BMMSCs to enhance their ability to induce differentiation [15,16], increase their activity, and prolong their duration of action [17].

Since both BMMSCs and HO-1 have reparative effects on transplanted liver, and since HO-1 acts synergistically with BMMSCs to protect the allograft [14], we used genetically modified adenovirus as a carrier to transduce the rat HO-1 gene into BMMSCs ex vivo to form Adv-HO-1/BMMSCs. We then observed the effects of these altered stem cells on lymphocyte proliferation and cytokine secretion in vitro. The immunomodulatory effects of Adv-HO-1/BMMSCs on the reduced-size transplanted liver were also studied in the rat rejection model.

2. Materials and methods

2.1. Main instruments and reagents

The following instruments and reagents were used: Dulbecco's modified Eagle medium (DMEM)/F12 (Hyclone, Logan, UT, USA); fetal bovine serum (PAA, Austria); glutamine and penicillin-streptomycin solution (Gibco, Carlsbad, CA, USA); trypsin, dimethyl sulfoxide (DMSO), and phosphate buffer solution (Sigma-Aldrich, St. Louis, MO, USA); red blood cell (RBC) lysis buffer (10×; BD Biosciences, San Jose, CA, USA); enzyme-linked immunosorbent assay (ELISA) kits for IL-10, TGF-β, IL-2, IL-17, IL-23, and TNF-α in rats (Santa Cruz Biotechnology, Dallas, TX, USA); recombinant adenovirus expressing rat HO-1 (Genechem Co., Ltd., Shanghai, China); lymphocyte separation medium, methylthiazolyl tetrazolium (MTT), and Oil Red O powder (Dingguo Changsheng Biotechnology, Beijing, China); Con A (Sigma, Germany); von Kossa cell staining kit (Genmed Gene Pharmaceutical Technology Co., Ltd., Shanghai, China); polyclonal rabbit anti-rat HO-1 antibody (Stressgen Biotechnologies, Victoria, BC, Canada) and goat anti-rabbit IgG secondary antibody; RNAiso Plus RNA extraction reagent, PrimeScript II first-strand cDNA synthesis kit, and SYBR Premix Ex Taq (TaKaRa, Japan); flow cytometry–related antibodies CD45/CD29/RT1A-PE, CD90/CD34/RT1B-FITC, CD3-APC, and CD25/CD71-FITC (BioLegend, San Diego, CA, USA); Transcription Factor Buffer Set, CD4-FITC, CD25-PE, FoxP3-PE-Cyanine5, CD3-APC, and IFN-γ-FITC (eBioscience, San Diego, CA, USA); CD161-PE (BD, NJ, USA); PMA (Sigma, CA, USA); ionomycin and brefeldin A (Yeasen Co., Ltd., Shanghai, China); fluorescence-based ABI 7300 quantitative real-time polymerase chain reaction (qRT-PCR) detection system (Applied Biosystems, Foster, CA, USA); YAC-1 cells (Peking Union Cell Line Resources, Beijing, China); RPMI 1640 medium; Hank's balanced salt solution (pH 7.2–7.4) and 1% NP40 (Gibco, NY, USA); and sodium lactate, nitroblue tetrazolium chloride (NBT), methylphenazinium methosulfate (PMS), nicotinamide adenine dinucleotide trihydrate (NAD) I, citrate, and trypan blue (Solarbio, Beijing, China).

2.2. Animals

Specific-pathogen-free adult inbred male Brown-Norway (BN) rats and male Lewis rats were purchased from Vital River Laboratory Animal Technology Co. Ltd., Beijing, China. The rats were housed individually in standard animal facilities at 24 °C–30 °C with a 12-h light/dark cycle and were provided commercially available chow and tap water *ad libitum*. Inbred male Lewis ($RT1^l$) rats (210–250 g; 8–10 weeks old) were the liver transplantation donors, and inbred male BN ($RT1^n$) rats (210–250 g; 8–10 weeks old) were the recipients. The difference of body weight between donor and recipient of each pair did not exceed 10 g. BMMSCs were extracted from syngeneic inbred male BN rats (80–100 g; 4–5 weeks old).

The study protocol was approved by the Animal Care and Research Committee of Tianjin First Central Hospital, Tianjin, China. Chloral hydrate anesthesia was used when the rats were operated upon or killed. Every effort was made to minimize animal suffering.

2.3. Isolation of BMMSCs and transduction of BMMSCs with HO-1-bearing recombinant adenovirus

2.3.1. Isolation, culture, and characterization of BMMSCs

BMMSCs were isolated aseptically from the femur and tibia of syngeneic male BN rats after the animals were killed by cervical dislocation. After cutting off both epiphyseal ends of the bones, the marrow cavity was rinsed out with DMEM/F12 containing 10% fetal bovine serum (FBS). RBCs were lysed using 0.1 mol/L NH_4Cl, and the remaining cells were washed, resuspended, and cultured at 37 °C with 5% CO_2 in DMEM/F12 containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 15% FBS. The medium was changed 48 h later, and the nonadherent cells were removed. Thereafter, the medium was changed every 72 h. When the cells reached 80% confluence, they were passaged in a ratio of 1:2. The well-grown third-passage cells were fluorescent-labeled with antibodies against CD34, CD29, CD45, CD90, RT1A, and RT1B (Santa Cruz Biotechnology, Dallas, TX, USA) for flow cytometric analysis of the expression of cell surface markers [18]. The third-passage BMMSCs were also inoculated in 6-well plates at 2×10^5/well and cultured in adipogenic differentiation medium (DMEM/F12 containing 10% FBS, 1 μM dexamethasone, 10 μg/mL insulin, 0.5 mM 1-methyl-3-isobutyl xanthine, and 0.1 mM indomethacin) or osteogenic differentiation medium (DMEM/F12 containing 10% FBS, 0.1 μM dexamethasone, 10 mM sodium glycerophosphate, and 50 μg/mL vitamin C). The medium was changed every 72 h. BMMSCs were identified by Oil Red O staining 2 weeks after induction or von Kossa staining 3 weeks after induction.

2.3.2. Transduction and identification of BMMSCs with HO-1-bearing recombinant adenovirus

HO-1-bearing recombinant adenovirus (adenovirus/HO-1) was diluted to 10 pfu/cell with complete culture medium, and this was used to replace the original medium of the BMMSCs. After 6–8 h, the adenovirus/HO-1 culture medium was replaced by complete culture medium for continued cultivation of the BMMSCs. After 48 h, the infection efficiency was assessed by evaluating the proportion of cells displaying green fluorescence indicative of infection. Then the total intracellular RNA was extracted, and the expression of intracellular HO-1 gene and endogenous reference gene β-actin was analyzed by fluorescence quantitative RT-PCR; the expression difference of HO-1 was calculated by relative quantitative analysis. The PCR primer sequence was as follows: HO-1-S, 5′ CTGGCTCTCTTTTCTTGG 3′; HO-1-AS, 5′ ATGGTCAGTCAACATGGAC 3′; β-actin-S, 5′ GCGTGACATTAAAGAGAAGCTG 3′; β-actin-AS, 5′ AGAAGCATTTGCGGTGCAC 3′.

The third-passage well-grown BMMSCs were trypsinized, counted, centrifuged, and resuspended, and then cultured in a T75 culture flask. After BMMSCs became completely adherent, Adv-GFP and Adv-HO-1 were added into the flask, away from light

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