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TGF-β expression by allogeneic bone marrow stromal cells ameliorates diabetes in NOD mice through modulating the distribution of CD4+ T cell subsets

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ABSTRACT

BMSCs could promote the regeneration of islet β-cell, but the status of BMSCs under diabetes is still unknown. Our study verified the effect of allogeneic BMSCs (ICR) transferred into NOD mice on blood glucose and CD4+ T cells subsets function. In vivo experiment, BMSCs could decrease blood glucose, weaken lymphocytes proliferation. In vitro experiment, the distribution of CD4+ T cell subsets was changed after co-culture with BMSCs, resulting in a greater frequency of Treg cells and reduced representation of Th17 cells. After TGF-β blockade, CD4+ T cells differentiated along a route favoring development of Th17, but not Treg cells. Thus, NOD can be treated by BMSCs which changes the distribution of CD4+ T cells, increases the number of Treg cells, and inhibits the differentiation of Th17 cells. And the positive effects of allogeneic BMSCs in the treatment of NOD mice depend on the regulation of TGF-β secreted by BMSCs.

1. Introduction

BMSCs, which have the potential for self-renewal, are regarded as multipotent stem cells that can differentiate into multiple mesenchymal and non-mesenchymal lineages, including osteocytes, chondrocytes, myocytes, and adipocytes [1]. Type 1 diabetes is a T cell mediated autoimmune disease in which an imbalance of Th1 and Th2 cells is thought to play an important role in the pathogenesis of disease [2]. CD4+ T cells were once subdivided into only two independent subsets, Th1 and Th2 cells; recently however, other CD4+ T cell populations have been described, such as Treg and Th17 cells, and their essential roles have been identified in autoimmune diseases. Previous studies demonstrated that Treg and Th17 cells are associated with the pathogenesis of autoimmune disease [3,4]. It is still unknown if the balance of Treg and Th17 cells is likely a function in autoimmune disease therapy. The differentiation potency of BMSCs has been emphasized in earlier studies, and BMSCs have emerged as a promising tool for regeneration and tissue engineering. However, BMSCs have also recently been demonstrated to suppress many T-cell, B-cell, and NK-cell responses, and may affect dendritic cell activity, as well, thus exerting an immunoregulatory capacity both in vitro and in vivo [5]. Based on these properties, BMSCs transplantation for experimental autoimmune disease therapy has been increasingly widely applied.

Most of studies of BMSCs role in diabetes had used syngeneic cells. Few studies used allogeneic cells. However, BMSCs could hardly be recognized by the host and escape the immunological rejection. This could be contributed to the lack of costimulatory molecules such as CD80, CD86, and CD40. In addition, allogeneic BMSCs could elicit more significant T cell inhibition [6]. In our study, we chose the allogeneic BMSCs of ICR mice as the transplanted cells to investigate the immunomodulation effects of BMSCs on NOD mice.

So far, it has been confirmed that BMSCs inhibitory effect mediated through various soluble cytokines [7,8]. One of these cytokines is TGF-β. It has been demonstrated that TGF-β responsible for the augmentation of BMSCs inhibitory effect in cultures with direct contact [9]. In addition TGF-β affects Treg T cell differentiation, down-regulates the function of Th1 and Th17 cells, and ameliorates autoimmune disease [10]. Suppression of insulitis in non-obese diabetic (NOD[2]) mice by oral insulin administration was associated with selective expression of TGF-β [11]. In another study, significant protection was mediated by transgenic expression of TGF-β by islet infiltrating Treg cells [12], indicating that the suppressive activity of TGF-β depended upon CD4+CD25+ T cells. However, it is now often difficult to distinguish true Treg cells from activated CD4+ non-regulatory T cells using CD25 [13]. FoxP3 is currently the most specific Treg marker known and CD4+Foxp3+ T cells were used in our study to facilitate the study of Treg cells.

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[2] *Abbreviations used:* NOD, non-obese diabetics; BMSCs, bone marrow stromal cells; H&E, hematoxylin and eosin; cpm, counts per minute.

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The purpose of this study was to investigate the therapeutic effect of intravenous administration of allogeneic BMSCs on NOD diabetes, to clarify the contribution of allogeneic BMSCs in the treatment of diabetes in NOD mice in vivo, and to determine their effects on the imbalance of CD4+ T subsets in vitro. In the current study, we wished to investigate our suspicion that the therapeutic effect of BMSCs was realized through the secretion of TGF-β.

2. Materials and methods

2.1. Mice

Female NOD/Lt mice at 12 weeks of age and ICR mice at 6–8 weeks were purchased from Shanghai SLAC Laboratory Animal Co. and maintained under specific pathogen-free conditions at 21 ± 2 °C and 45 ± 5% humidity. All animal handling and experimental procedures were approved by the Animal Care and Use Committee of Chinese Academy of Medical Sciences. Female NOD/Lt mice were tested for blood glucose twice a week and were considered to have diabetes if blood glucose levels were above 250 mg/dl for two sequential blood glucose tests. In our laboratory, the incidence of diabetes in female NOD/Lt mice at 30 weeks of age reached 75%. NOD mice when their blood glucose levels were between 250 and 400 mg/dl were chosen for BMSCs transplantation. Eight mice for each experimental group and six mice for control group.

2.2. Islet isolation

Islets of Langerhans were isolated from mice as previously described [14]. Briefly, the pancreas was perfused via the common bile duct with collagenase P (Roche Molecular Biochemicals, Indianapolis, IN) followed by purification of islets on a Histopaque-1077 gradient (Sigma–Aldrich, St. Louis, MO). Islets were dispersed into single cells by brief incubation with 0.2% trypsin (Calbiochem, San Diego, CA)/10 mM EDTA in HBSS. Dispersed islets were then washed free of trypsin and cultured in RPMI 1640 + 10% FCS. Islet cells were usually used on the day of isolation.

2.3. Preparation and transplantation of BMSCs

BMSCs were obtained from 6- to 8-week-old ICR mice. The animals were killed by cervical dislocation and the bone marrow was flushed out of tibias and femurs. After washing by centrifugation at 400*g* for 10 min and counting viable BMSCs with trypan blue, the BMSCs were re-suspended in stem cell culture medium (MesenCult basal medium plus Mesenchymal Stem Cell stimulatory supplements; Stem Cell Technologies, CA) to a final concentration of 1×10^6 viable cells per ml. The culture was kept in a humidified 5% CO_2 incubator at 37 °C for 72 h, at which time non-adherent cells were removed by changing the medium. Medium was changed every 4–5 d until clones grew out.

A total of 5×10^6 donor BMSCs suspended in 0.1 ml PBS were transferred intravenously via the tail-vein into NOD mice (blood glucose between 250 and 400 mg/dl). Mice of the control group were injected PBS. Blood glucose was monitored every other day after transplantation on tapped tail-vein blood with a portable glucose monitor (ACCU SOFT, Canada).

2.4. Evaluation of insulitis and histological analysis

Pancreata from NOD mice sacrificed 35 days after BMSC transfer were fixed in 4% paraformaldehyde solution, dehydrated, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E), and duplicated sections were stained with rat anti-mouse insulin (1:200; Santa Cruz, USA) or rabbit anti-mouse IL-17 (1:200; Santa Cruz), followed by biotinylated goat anti-rat IgG or biotinylated goat anti-rabbit IgG and DAB substrate for peroxidase. All sections were counterstained with hematoxylin. Tissue areas were measured from 200× or 400× magnification digital photos using Image Pro Plus software (Media Cybernetics, Silver Springs, MD) and a reference stage micrometer scale also captured at 200× or 400× power. The severity and extent of insulitis were scored using the following classification: 0, no mononuclear cell infiltration; 1, cell infiltration < 25%; 2, cell infiltration 25–50%; 3, cell infiltration 50–75%; 4, cell infiltration > 75%.

2.5. Proliferation assay

2.5.1. In vivo

Eight mice chosen from BMSCs group and control group were sacrificed at the end of observation (days 35 post cells transfer). Single cell suspensions from pancreatic lymph nodes were prepared and cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine (Sigma, Santa Clara, CA, USA), 0.1 mM non-essential amino acids, 5×10^{-5} M 2-mercaptoethanol (2-ME; Amresco, Solon, OH, USA), 1 mM sodium pyruvate, and 100 IU/ml penicillin, 100 μg/ml streptomycin (Gibco, Paisley, UK). Samples (200 μl) of mononuclear cell (MNC) suspensions were applied in triplicate into 96-well round-bottomed wells of microtiter plate (Nunc, Copenhagen, Denmark) at a cell density of 2×10^6 cells/ml. At the same time, 5×10^5 irradiated spleen cells (20 Gy, ^{60}Co source) and 2×10^4 islet β cells both isolated from NOD mice of 6–8 weeks ages were added together to per well. After 60 h of incubation, the cells were labeled for an additional 12 h with 10 μl solution containing 1 μCi ^{3}H-methelthymidine (specific activity, 60 Ci/mmol; China Institute of Atomic Energy, Beijing). Cells were harvested onto glass fiber filters and thymidine incorporation was measured in a liquid β-scintillation counter (Perkin-Elmer, Wellesley, MA). The results were expressed as counts per minute (cpm) per culture.

2.5.2. In vitro

Pancreatic lymph nodes were separated aseptically from NOD mice with blood glucose levels between 250 and 400 mg/dl and then single cell suspensions were prepared. MNCs (4×10^5), 5×10^5 irradiated spleen cells and 2×10^4 islet β cells mixed in 200 μl were applied in triplicate into 96-well round-bottomed wells of microtiter. Where indicated, BMSCs irradiated with 30 Gy were added to the co-cultures (at the proportion of 1:10, compared with β-cell-specific T cells). Where indicated, 10 μg/ml TGF-β neutralizing antibody or isotype control IgG was added into the culture system. After incubation, cells were labeled with ^{3}H-methelthymidine and measured by β counter.

2.6. Measurement of cytokines

2.6.1. In vivo

NOD mice with blood glucose between 250 and 400 mg/dl were sacrificed by cervical dislocation 35 days with or without BMSCs transplantation, and the serum was collected for IL-17, TGF-β, IL-6, IFN-γ, IL-4, and IL-10 detection by ELISA according to the manufacturer's instructions (Jingmei Biotech Co., Ltd., Beijing, China).

2.6.2. In vitro

Pancreatic lymphocytes were prepared from six NOD mice (blood glucose level between 250 and 400 mg/dl) and cultured alone or with irradiated ICR BMSCs (30 Gy, ^{60}Co) at the proportion of 10:1. Where indicated, neutralizing anti-TGF-β antibody (10 μg/ml) or isotype control IgG was added into the culture system. After 72 h, supernatants from different cell culture systems were collected for IL-17, TGF-β, IL-6, IFN-γ, IL-4, and IL-10 detection.