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ORIGINAL ARTICLE

Effects of Bone Marrow Mesenchymal Stem Cells on Plasminogen Activator Inhibitor-1 and Renal Fibrosis in Rats with Diabetic Nephropathy

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Background and Aims. We undertook this study to observe the effects of bone marrow mesenchymal stem cells (BMSCs) on plasminogen activator inhibitor-1 (PAI-1) and renal fibrosis in rats with diabetic nephropathy and to explore its main mechanism.

Methods. Thirty male Sprague Dawley rats were randomly divided into three groups: normal control group (NC group, $n = 10$), diabetic nephropathy group (DN group, $n = 10$), stem cell transplantation group (MSC group, $n = 10$). BMSCs were transplanted to rats in the MSC group via caudal vein infusion (2×10^6/mL). At the end of 12 weeks, blood glucose, 24-h urinary protein, serum creatinine and renal mass index were measured. Morphology and collagen deposition in rat kidney were observed by HE and Masson staining, respectively. Expressions of PAI-1, transforming growth factor β1 (TGF-β1) and Smad3 in rat kidney were detected by immunohistochemistry and Western blot.

Results. Compared with DN group, 24-h protein, serum creatinine and renal mass index decreased significantly in MSC group. No significant changes in blood glucose ($p > 0.05$) were shown. Immunohistochemistry and Western blot showed that expressions of PAI-1, TGF-β1 and Smad3 in NC group were lower than DN group. Expression of each protein in MSC group was between two groups ($p < 0.05$). Correlation analysis revealed that PAI-1 and TGF-β1 ($r = 0.987$, $p < 0.05$) and Smad3 ($r = 0.974$, $p < 0.05$) showed a significant positive correlation. TGF-β1 and Smad3 ($r = 0.962$, $p < 0.05$) were positively correlated.

Conclusions. BMSCs significantly inhibited renal fibrosis in rats with DN. The mechanism may be related to inhibition of TGF-β1/Smad3 pathway, decreasing the expression of PAI-1 protein and reducing the accumulation of extracellular matrix, thereby balancing the fibrinolytic system.

Key Words: Bone marrow mesenchymal stem cells, Diabetic nephropathy, Renal fibrosis, Plasminogen activator inhibitor-1, Transforming growth factor-β1/Smad3 signaling pathway.

Introduction

Diabetic nephropathy (DN) is a major complications of diabetes and represents the leading cause of end-stage renal disease worldwide (1). Renal fibrosis is an independent factor in the process of DN. Delaying the process of renal fibrosis plays an important part in preventing the process of DN (2). Accumulation of extracellular matrix (ECM) is the ultimate pathway to renal fibrosis, and plasminogen and plasmin system are important in the regulation of the dynamic equilibrium of ECM (3). Studies have shown that a rat model without PAI-1 gene was slower than the normal group in the process of renal fibrosis, which indicates the important role of PAI-1 in DN renal fibrosis (4). Research also showed that increased expression of PAI-1 inhibited degradation of glomerular and tubular ECM, which resulted in progressive accumulation of ECM, thickened the

Conflict of Interest: The authors declare that they have no conflict of interest.

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glomerular basement membrane, and accelerated glomerular sclerosis (5). Another study found that inhibition of TGF-β1 (TGF-β1)/Smad3 signaling pathway can reduce accumulation of ECM and significantly improve fibrosis, thus protecting renal function in a number of disease states (6). In recent years, theory and technology of stem cells has been developing rapidly. Studies have shown that proteinuria of diabetic animal models transplanted with bone marrow mesenchymal stem cells (BMSCs) was significantly reduced (7). However, it is not clear if BMSCs can suppress the TGF-β1/Smad3 signaling pathway, decrease PAI-1, eventually reduce accumulation of ECM and delay renal fibrosis. In this study we explore this issue through the establishment of STZ-induced DN model.

Materials and Methods

Experimental Animals

Thirty-eight 8-week-old male Sprague Dawley rats weighing 200−250 g were purchased from the Experimental Animal Centre of Xuzhou Medical College.

Experimental Materials

Streptozotocin (STZ, Sigma, St. Louis, MO), fetal bovine serum (Hangzhou Evergreen Company, China), DMEM/F12 medium (Beijing Thermo Company, Bejing), anti-mouse CD90-PE (BioLegend, San Diego, CA), anti-mouse CD29-FITC, anti-mouse CD45-FITC (eBioscience Corporation, San Diego, CA), rabbit polyclonal anti-rat PAI-1 antibody (Wuxi Pharmaceutical Biotechnology Co. Ltd. China), rabbit TGF-β1 polyclonal anti-rat antibody (Abcam, UK), rabbit polyclonal anti-rat Smad3 antibody (Abcam), PV-9002 rabbit two-step detection kit (Beijing Zhongshan Golden Bridge Co. Ltd., China), and IR-DyeTM800 (LI-COR, Lincoln, NE) labeled goat anti-rabbit antibody were used.

Isolation, Culture and Passing of BMSCs

Male Sprague Dawley rats weighing 80−120 g were killed by cervical dislocation and soaked with 75% ethanol for 20 min. Femur and tibia were removed under sterile conditions, exposing the bone marrow cavity. Next, rats were washed repeatedly in a narrow chamber with PBS using a 5 mL syringe and fluid was collected into a sterile centrifuge tube and centrifuged for 5 min at 1000 rpm. Then, 4 mL DMEM/F12 medium containing 10% fetal bovine serum was added into a sterile centrifuge tube, the supernatant was removed and finally placed into a single cell suspension. These were inoculated into 25 cm^2 flasks at 37°C and placed into 5% CO_2 incubator with thermostat. The first half of the medium was changed after 48 h. Medium was changed once every 3 days until cell fusion increased from 80−90%, with 0.25% trypsin at a 1:2 ratio for cell subculture. Cell morphology was observed daily under an inverted microscope.

Identification and Labeling of MSCs

Flow Cytometry

The third generation of BMSCs grew optimally. After trypsin digestion, cells were collected in the flow tube and the centrifuged supernatant was discarded. After the cells in the test samples were added to the anti-mouse CD90-PE, anti-mouse CD29-FITC, CD45-FITC anti-mouse antibody dilutions each 10 μl (blank tube without antibodies, the remaining steps were the same) were incubated in the dark at room temperature for 30 min, washed twice with PBS, centrifuged, supernatant discarded and, finally, 1% paraformaldehyde (200 μl) was added.

BMSCs In Vitro *Labeling*

Taking the third generation of BMSCs that grew optimally, cells reached 80−90% confluence. BrdU containing 10% serum was added to a final concentration of 10 μm mol/L of DMEM/F12 medium, 37°C, 5% CO_2 and incubated for 3 days. Cells were collected within 30 min before transplanting into MSC rat group, placing them into suspension cells with normal saline.

Animal Model and Experimental Groups

Sprague Dawley rats were divided into normal control group ($n = 10$) and model group ($n = 28$) with a random number table. After adaptive feeding for 1 week and fasting 12 h, the model group was given STZ55 mg/kg by i.p. injection. Control group was given the same amount of citrate buffer by i.p. injection. Tail vein blood was collected after 72 h. Random blood glucose test was >16.7 mmol/L, which proved that the model was a successful diabetes model. Six model rats died. Two rats did not meet the standards during the experimental study. Model rats were randomly divided into two groups: diabetic nephropathy group ($n = 10$) and stem cell transplantation group ($n = 10$). For rats in the MSC group, intravenous (i.v.) infusion of autologous MSCs (2×10^6/mL) was performed 4 weeks after the onset of diabetes via the tail vein. Animals in the vehicle group received an equal volume of culture medium at the same time.

Collection and Sample Testing

At 12 weeks, rats were placed in metabolic cages to collect and detect 24 h urinary protein. Blood glucose, body weight and abdominal aorta blood were detected. Serum of abdominal aorta blood was measured for serum creatinine (Scr). All rats were then sacrificed by decapitation and their