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Research Article

Effect of erythropoietin on the migration of bone marrow-derived mesenchymal stem cells to the acute kidney injury microenvironment

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ABSTRACT

Bone marrow-derived mesenchymal stem cells (BMSCs) preferentially migrate to the injured tissue but with limited efficiency. Here we investigated the effect of erythropoietin (EPO) treatment on the BMSC migration to the acute kidney injury (AKI) microenvironment. The possible mechanisms were also discussed. A hypoxia/re-oxygenation (HR) model of renal tubular epithelial cells (RTECs) was established to generate AKI in vitro, and a chemotaxis experiment was conducted using the transwell chamber. EPO treatment enhanced the BMSC migration to the HR-RTEC culturing chamber in a SDF-1 level-dependent manner, which was fully inhibited by the treatment of anti-SDF-1 antibody. The BMSC migration could also be partly blocked by LY294002 (phosphoinositide 3-kinase (PI3K) inhibitor) and PD98059 (MAPK inhibitor). Western blot analysis showed that phosphorylated Akt and phosphorylated MAPK in BMSCs were enhanced by EPO treatment. In the in vivo experiment, BMSCs were transplanted into the AKI mice and EPO was subcutaneously injected. The results showed that EPO injection increased the SDF-1 protein expression and BMSC accumulation in the renal tissue, which was consistent with a decent improvement of renal function. In addition, the BMSC accumulation in the renal tissue was blocked by anti-SDF-1 antibody, LY294002 or PD98059. Our data suggest that AKI microenvironment had a directional chemotactic effect on BMSCs, which could be further enhanced by the EPO treatment. The increased SDF-1 level in the AKI microenvironment and the activations of PI3K/AKT and MAPK in BMSCs were the possible mechanisms for the effect of EPO. Therefore, BMSC transplantation combined with EPO injection can be a novel and effective approach for AKI repair.

Introduction

Acute kidney injury (AKI) is a common clinical disease and the prognosis and mortality are still significant issues. Previous studies [1–6] have suggested that transplantation of bone marrow-derived mesenchymal stem cells (BMSCs) is helpful for the repair of AKI because of their preferential migration to the injured kidney and paracrine/autocrine mechanisms or differentiation potential. However, many transplanted BMSCs can still stay in the blood-rich organs. Therefore, to maintain the repair effect

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when a limited number of BMSCs is transplanted, it is vital to increase the efficiency of the BMSC migration to the injured kidney. Erythropoietin (EPO) is a glycoprotein that has been suggested to be effective in treating AKI [7–9]. Although it has been suggested that EPO has positive effects on the BMSC migration [10,11], its effect on the kidney-directional migration of BMSCs remains unclear.

In this study, a hypoxia/re-oxygenation (HR) model of renal tubular epithelial cells (RTECs) was established to generate AKI in vitro, and the effect of EPO treatment on the BMSC migration to the AKI microenvironment was investigated. Furthermore, AKI mouse models were constructed and the effect of EPO was further investigated through the in vivo experiment. The possible mechanisms were also explored.

Materials and methods

Construction of the HR model of RTECs to generate AKI in vitro

Mouse RTECs, which were purchased from American Tissue Culture Collection (ATCC) (Manassas, VA, USA), were divided into 5 groups: the Control Group, the H_6/R_{12} Group, the H_{12}/R_{12} Group, the H_{16}/R_{12} Group and the H_{24}/R_{12} Group. For the Control Group, 4×10^5/well RTECs were cultured in the 6-well plates under the normoxia condition (95% air and 5% CO2) with low-glucose DMEM (Invitrogen, San Diego, CA, USA). For the other 4 groups, 4×10^5/well RTECs were cultured in the 6-well plates first under the hypoxia condition (3% O_2, 5% CO_2 and 92% N_2) using the AnaeroPack™ System (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) with glucose-free DMEM (Invitrogen) for 6 h, 12 h, 16 h and 24 h, respectively. Then, they were all cultured under the re-oxygenation condition (95% air and 5% CO_2) with low-glucose DMEM for 12 h.

The general changes of cell morphology in the 5 groups were monitored. An Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime Biotechnology Co., Ltd., Nanjing, China) was used to evaluate the cell apoptosis. Briefly, after being rinsed with ice-cold PBS, the cells were resuspended in 200 μl binding buffer. After 5 μl Annexin V-FITC was added, the cells were incubated at room temperature for 10 min. The cells were then further incubated with 10 μl propidium iodide, followed by an instant analysis using FACScan.

According to the changes of cell morphology and the apoptosis rate of RTECs, the group that can maximally generate AKI in vitro was determined, based on which HR-RTECs were prepared for further use. RTECs (4×10^5/well) cultured totally under the normoxia condition, whose culturing time was consistent with the hypoxia/re-oxygenation time used for HR-RTECs, were selected as the control.

Chemotaxis assay

The chemotaxis experiment was conducted using the transwell chamber with an 8-μm pore size polycarbonate filter (Coring Incorporated, NY, USA). Upon completion of the preparations of HR-RTECs and RTECs, the 6-well plates were removed from the incubator and the transwell chambers were inserted into the plates. Mouse BMSCs (ATCC) resuspended in the medium were plated in the upper chambers and the plating density was 1.8×10^5/chamber. The chemotaxis assay was used for the following groups: the Control group (BMSCs/RTECs), the HR group (BMSCs/HR-RTECs) and the EPO groups (different concentrations (1 IU/ml, 5 IU/ml, 10 IU/ml, 50 IU/ml) of recombinant human EPO (rhEPO, Roche, China) were added into the HR-RTEC culture medium, then, the transwell chambers were inserted into the plates and BMSCs were added into the upper chambers).

Stromal cell-derived factor 1 (SDF-1) inhibition experiment was also performed. 10 IU/ml rhEPO and 5 μg/ml anti-mouse SDF-1 monoclonal antibody (R&D systems, Wiesbaden, Germany) were both added into the HR-RTEC culture medium. For the other two inhibition experiments, the BMSCs in the EPO (10 IU/ml) group were first preincubated with 20 μM LY294002 (phosphoinositide 3-kinase (PI3K) inhibitor) or 5 μM PD98059 (MAPK inhibitor) (both were purchased from Sigma-Aldrich, St Louis, MO, USA) for 30 min and then added into the upper chambers. All the groups were then incubated at 37 °C for 6 h in a humidified atmosphere with 5% CO_2. After completion of the co-culturing, the nonmigrating BMSCs from the upper surface of the polycarbonate membrane were wiped out with a cotton bud. The BMSCs under the membrane were stabilized with 4% paraformaldehyde for 20 min and stained with 1% crystal violet for 30 min. In the final step, PBS cleaning was carried out till the violet color became dim. Photographs were taken for each membrane under the microscope, and the numbers of cells in 5 non-overlapped visual fields were counted and the average number of migrating cells was calculated.

SDF-1 levels in RTECs or HR-RTECs

***Protein expression*:** After 6 h co-culturing, the transwell chambers were removed. Cell lysate was added into the 6-well plates for the full lysis of RTECs (or HR-RTECs). The supernatant was taken after centrifugation and the protein concentration was measured. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the protein was transferred to the PVDF membrane. After being sealed, the PVDF membrane was incubated with rabbit anti-mouse SDF-1 monoclonal antibody at 4 °C overnight. HRP labeled goat anti-rabbit IgG (Santa Cruz Biotechnology Inc., CA, USA) was added and the membrane was incubated for 1 h at room temperature. The mixture of the reacted ECL was added to the PVDF membrane for 1–2 min, and then the PVDF membrane was placed into the Fluorchem HD2 gel image analysis system for analysis. The intensity of the band was measured. Tubulin was used as the internal reference. The protein expression was the ratio of the two band gray values.

***Supernatant concentration*:** We removed the transwell chambers, and the culture supernatants of RTECs and HR-RTECs in the 6-well plates were collected to measure the SDF-1 concentration using the SDF-1 Enzyme-linked Immunosorbent Assay (ELISA) Detection Kit (Round Record Biotech Co., Ltd, Shanghai, China) according to the manufacturer's instructions.

Phosphorylations of AKT and MAPK in BMSCs

After completion of the co-culturing, the transwell chambers were transferred to the new blank 6-well plates, and cell lysate was added for the full lysis of BMSCs. Western blot was employed as described above with rabbit anti-mouse phospho-Akt (pAKT)

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