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Erythropoietin-activated mesenchymal stem cells promote healing ulcers by improving microenvironment

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ABSTRACT

Background: Mesenchymal stem cell (MSC) transplantation is an effective treatment therapy for ischemic ulcers. However, in high-glucose microenvironment, the original inflammation-inhibiting function of MSCs leads to turns into secreting large amounts of inflammatory mediators, such as tumor necrosis factor alpha, for example, which decreases their capacity and becomes poor quality stem cells over inflammation cells for diabetic foot ulcers repair in the healing of diabetic foot ulcers. Erythropoietin (EPO) is an anti-inflammatory, proangiogenic cytokine. It is unclear whether EPO-activated MSCs with biomaterials can promote the effective healing of diabetic foot ulcers.

Methods: Cultivated MSCs in MSC-L, MSC-H, EPO-G, Akt-G, and mTOR-G, then separated the supernatant-conditioned medium of these groups to stimulate human umbilical vein endothelial cells on proliferation and migration experiments; a new type of biomaterial planted with the EPO-activated MSCs was applied to the diabetic foot ulcers of the C57 mice.

Results: In vitro experiments showed that EPO could stimulate MSCs to secrete vascular endothelial growth factor in high-glucose microenvironment. More importantly, EPO could reduce the damage to MSCs by high-glucose microenvironment, promote their proliferation and migration functions, and inhibit the high-glucose–induced MSCs from secreting the inflammatory mediator tumor necrosis factor alpha. *In vivo* experiments showed greater angiogenesis in EPO-MSC group than in control group, ulcer healing in EPO-MSC group was significantly better than that in control group, and MSCs partially differentiated into endothelial cells. EPO-activated MSCs could inhibit the monocyte invasion of localized diabetic foot ulcers.

Conclusions: Our results indicate that EPO-activated MSCs can promote the effective healing of diabetic foot ulcers. The mechanism is that EPO can change stem cells from excessive inflammation into general inflammation and improved diabetic foot ulcers inflammatory microenvironment.

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Introduction

It is anticipated that the worldwide prevalence of diabetes in adults will reach 5.4% by 2025, and the total number of diabetic adults will reach 300 million.[1] Among these, 1.0%-4.1% will experience diabetic foot ulcers to differing degrees, and the rates of lower limb amputation will be 0.21%-1.37%.[2]

Endothelial cells and mesenchymal stem cells (MSCs) are important in treating diabetic foot ulcers.[3,4] MSCs play the role of promoting angiogenesis and inhibiting inflammation in the skin wound as it heals. MSCs promote angiogenesis mainly through two ways: first, they secrete vascular endothelial growth factor (VEGF), which promotes the proliferation and migration of endothelial cells through paracrine pathways[5,6]; second, MSCs can differentiate into endothelial cells directly, thereby promoting revascularization in ulcerous tissue.[7,8] However, the current treatment results showed that MSC therapy to treat diabetic foot ulcers did not demonstrate the expected positive healing results that have been observed in other types of skin wounds.[9] This could be related to the high-glucose, hypoxic microenvironment of diabetic foot ulcers, which results in MSC dysfunction, reduced paracrine function and differentiation, and angiogenesis disorders.[10] It is important to note that high-glucose and anoxic microenvironment prevents MSCs from originally inhibiting tissue inflammation to secreting large amounts of inflammatory mediators such as TNF-a, which leads to enhanced local inflammation and results in healing disorders.[11]

EPO is a red blood cell regulating growth factor, which is mainly produced in the body by the kidneys. It can stimulate the proliferation and differentiation of erythroid progenitor cells. In particular, EPO's function is to protect the kidneys. Therefore, it is widely used in clinical treatment of a variety of anemia, such as neonatal and aplastic.[12] However, recent studies have found that the erythropoietin receptor (EPOR) is also widely distributed in various systems other than the kidneys, such as the brain. Therefore, EPO serves such functions as neuroprotection[13,14] antioxidation, anti-inflammation,[15] anti-apoptosis,[15,16] and so on. EPOR is also distributed in MSCs, but it is unclear whether EPO-activated MSCs can promote the healing of ischemic ulcers as is the mechanism by which they might do so. Therefore, we used EPO as a supplement to MSC therapy. Our aim was to reduce the negative stimulation of the MSCs from the high-glucose, hypoxic microenvironments, improve MSC function, and reduce the MSCs' secretions of inflammatory mediators. Thereby, we can change poor-quality MSCs to high-quality MSCs for the treatment of diabetic foot ulcers.

Due to the aforementioned findings, we planted EPO-activated MSCs on biomaterials, deposited the biomaterials on the diabetic foot ulcers of C57 mice, and studied the therapeutic effect.

Materials and methods

Cell culture

We prepared a α-MEM medium (Hyclone Laboratories) containing 10% fetal bovine serum, L-glutamine (2 mM; Hyclone Laboratories), penicillin (100 U/mL; Sangon.Com, China) and streptomycin (100 mg/mL; Sangon.Com, China) as the basic MSC medium. Separately, 5.6 mM of glucose was added to the basic MSC medium to form low-glucose microenvironment (LG) as MSC-L to simulate the normal blood glucose environment of body; 25 mM of glucose was added to the basic MSC medium to form high-glucose microenvironment (HG) as MSC-H to simulate the diabetic blood glucose environment of body[17]; we also added EPO (100 IU/mL; Sigma) to the MSC-H as the EPO group (EPO-G).[18] The Akt inhibitor Triciribine (5 μM; Kangchen.com, China) and mTOR inhibitor Rapamycin (5 μM; Sigma) were also added to the EPO-G, respectively as the Akt (Akt-G) and mTOR blocking groups (mTOR-G).[19,20] When contrasted the results among these groups, the MSC-L also as the control group in low-glucose microenvironment related to the MSC-H, EPO-G, Akt-G and mTOR-G, and the MSC-H as the control group in high-glucose microenvironment related to the EPO-G, Akt-G, and mTOR-G. We prepared an RPMI-1640 (Hyclone Laboratories, USA) medium containing 10% fetal bovine serum (Gibco), penicillin (100 U/mL) and streptomycin (100 mg/mL) as the basic human umbilical vein endothelial cells (HUVEC) medium. The MSCs and HUVECs were cultivated by basic MSC medium and basic HUVEC medium and placed in a 37°C, 5% CO_2 incubator. We observed the cell growth with an inverted microscope and used the logarithmic phase cells in the experiment.

MSC migration assays

We chose MSCs (GFP-transfected human bone marrow MSCs [Cyagen Biosciences, Guangzhou, China]) that were in the logarithmic phase. We then digested them with trypsin, prepared a cell suspension in an α-MEM basal medium, and counted the cells with an inverted confocal microscope. Then, we adjusted the cell density to 10^5/mL before planting the MSCs into 24-well plates. Next, 600-μL MSC-H, EPO-G, Akt-G, and mTOR-G were separately added to the 24-well plates. A 100-μL MSC suspension (1×10^4 cells) was added to the 25-mm 8.0-μm transwell chamber. We routinely cultured the cells in the incubator for 6 hours at 37°C, 5% CO_2. Then, we fixed them with 90% ethanol after removing the chamber, stained them with 0.5% crystal violet (Sigma) staining solution, and observed and photographed the cells, counting and calculating the average MSC numbers at four randomly selected low magnifications. We selected randomly four sampling points of four control groups (MSC-H), EPO-G, Akt-G, and mTOR-G at low magnification and counted the number of cells in each group sampling points of the average value.

Preparation of the MSC supernatant

We prepared MSCs that were in the logarithmic phase and planted them into six-well plates. Then, the cells were incubated with MSC-L, MSC-H, EPO-G, Akt-G, and mTOR-G for 48 h, respectively. Then, a supernatant-conditioned medium (CM) was filtrated with a 0.22-μm filter needle and preserved at −70°C, and these were labeled MSC-CM (LG), MSC-CM (HG), EPO-CM, Akt-CM, and mTOR-CM, respectively.[21] When contrasted the results among these groups, the MSC-CM (LG) as