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ORIGINAL ARTICLE

Zinc supplementation results in improved therapeutic potential of bone marrow-derived mesenchymal stromal cells in a mouse ischemic limb model

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Abstract

Background aims. We wanted to determine whether zinc supplementation can inhibit bone marrow-derived mesenchymal stromal cell (MSC) apoptosis and enhance their tissue regenerative potential a in mouse ischemic hindlimb model. *Methods*. Rat bone marrow cells were cultured and the resulting MSC were passaged for 3–7 generations. The proliferation and apoptosis of MSC was examined by 3-[4,5-dimethyl-2-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry analysis. The activation of protein kinases B (Akt) was determined by Western blots. Vascular endothelial growth factor (VEGF) levels were examined by enzyme-linked immunosorbent assay. The mouse hindlimb ischemic model was established by ligating the right femoral artery. Mice received MSC, zinc-treated MSC or vehicle. The blood flow was assessed by laser Doppler imaging. The survival rate of donor cells was quantified by real-time polymerase chain reaction for the sex-determining region of the Y-chromosome (Sry). Angiogenesis was assessed by histochemical staining and immunofluoresence staining. *Results*. Supplementation with physiologic amounts of zinc caused a marked attenuation of cell apoptosis, enhanced cell viabilities, increased VEGF release and up-regulated Akt activation. Zinc-treated MSC delivered into ischemic hindlimbs resulted in significant improvements in limb blood perfusion by increased implanted MSC survival and stimulated angiogenesis. *Conclusions*. This study demonstrates the potential of zinc supplement to enhance survival of engrafted MSC and ameliorate their tissue regenerative potential in a mouse ischemic hindlimb model.

Key Words: *angiogenesis, bone marrow stem cells, ischemic disease, zinc*

Introduction

Bone marrow is a source of stem cells and it can be employed for transplantation. Bone marrow-derived mesenchymal stromal cells (MSC) can differentiate into various tissues of mesenchymal origin (1,2), including stromal cells of marrow, adipocytes, osteocytes, chondrocytes (3,4), neurons (5), skeletal muscle and cardiac muscle (6). They have generated a great deal of interest in clinical application because of their potential use in regenerative medicine and tissue engineering. Bone marrow-derived MSC are a favorable source of cells required for transplantation because they are easily obtained and have high proliferation rates in cultures (7).

In spite of the potential advantages of bone marrow-derived MSC, the vast majority of implanted cells into ischemic tissues are believed to die within a few days of transplantation surgery, and most of this death is by apoptosis (8–10). The low therapeutic efficacy remains the largest obstacle to be overcome in stem cell therapy for angiogenesis. Because of a lack of initial vasculature, the hypoxic, inflammatory and pro-apoptotic environment in ischemic tissue causes poor survival of transplanted cells. Therefore,

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administration of cells alone may not have a significant enough effect on angiogenesis induction, and protection of MSC against apoptosis is critical for successful cellular therapy. Novel strategies are being developed to maximize biologic and functional properties of MSC, such as preconditioning of cells in culture (11), genetic transfection (9) and preparation of cells in special bioscaffolds (12). However, these strategies are complex and could have potential risk in clinical use. Given the increasing use of MSC in the treatment of a wide range of diseases, a relatively simple, safe and easily manipulated strategy to retain cell vitality is of great importance.

Zinc is an essential trace element that has a variety of physiologic roles in mammalian systems (13). Zinc itself is redox inert but it is associated with a large number of proteins (14). As such, zinc is an integral component of numerous metalloenzymes, structural proteins and transcription factors and contributes to physiologic processes including neurotransmission, hormone secretion, DNA synthesis and gene expression (15). In addition to these diverse physiologic roles, it is apparent that zinc is an inhibitor of many types of cells apoptosis (16–18) induced by diverse physical, chemical and immunologic stimuli. Previous studies have indicated that zinc deficiency may induce cell apoptosis, destroy tissue plasticity and depress tissue function (19,20). Compared with normal serum zinc concentrations, which average 12–18 μmol/L, cell culture media are usually very low in zinc, 0.7 pm–3 nm. Therefore, supplementing with zinc may prevent bone marrow-derived MSC from apoptosis, and pre-culturing MSC with a zinc supplement medium prior to transplantation could improve their tissue regenerative potential. We tested this hypothesis in a mouse ischemic hindlimb model, which presents a safe and easily manipulated clinical management option for retaining cell viability.

Methods

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Bethesda, MD, USA; NIH publication number 85–23, revised 1996). The Nanjing Medical University (Jiangsu, PR China) Animal Care and Use of Committee approved all animal protocols.

Cell cultures

MSC expansion was performed according to methods described previously (21,22). Under sterile conditions, with induction of general anesthesia by intraperitoneal injection with ketamine hydrochloride (2 mg/kg), bone marrow was aspirated from the femur and tibia of male rats using a 26-gauge needle, diluted in heparin (10 IU/mL) and incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Burlington, Ontario, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin. After centrifugation and resuspension, cells were cultured at 37°C, under 95% air–5% CO_2 conditions, in a gas-tight humidified incubator. Hematopoietic cells and other non-adherent cells were washed away during medium changes. The remaining purified MSC population was expanded further in cultures.

Measurement of vascular endothelial growth factor

To determine whether zinc pre-conditioning causes an increase in vascular endothelial growth factor (VEGF) release from MSC, a total of 1×10^4 MSC was plated in a serum-free medium with different doses of zinc (0, 0.1, 1, 10, 50 and 100 μmol/L) for 24 h. VEGF levels in conditioned medium were measured using VEGF enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. The results were compared with a standard curve constructed with murine VEGF (each assay carried out in triplicate for each group). Absorbance was measured at 450 nm by means of a microplate reader.

Cell proliferation assay

Cell proliferation was determined using a 3-[4,5-dimethyl-2-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MSC were plated on 12-well plates (1×10^4 cells) and cultured in a serum-free medium for 24 h with 10 μmol/mL zinc or blank control. Cells were washed gently with warm phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+}. Subsequently 90 μL DMEM without phenol red or FBS and 10 μL MTT solution (2 mg MTT/mL PBS without Ca^{2+}/Mg^{2+}) were added to each well and incubated for 4 h at 37°C. The MTT-containing solution was discarded and the formazan crystals dissolved in 75 μL 0.04 M HCl in isopropanol with shaking for 5 min at room temperature. The absorbance at 540 nm was measured using a microplate reader.

Cell apoptosis assay

Cells were incubated with or without zinc (10 μmol/L) for 24 h; cultures were then exposed to H_2O_2 (100 μmoL) for 60 min to induce apoptosis. The number of apoptotic cells after H_2O_2 exposure was evaluated by double staining with fluorescein isothiocyanate (FITC) Annexin V and propidium iodide (PI), and quantified by flow cytometric analysis (FACS Scan; Becton Dickinson, San Jose, CA, USA). Cell total proteins were then collected for Western blotting analysis.