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Bone marrow-derived mesenchymal stromal cells improve vascular regeneration and reduce leukocyte-endothelium activation in critical ischemic murine skin in a dose-dependent manner

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Abstract

Background aims. Stem cells participate in vascular regeneration following critical ischemia. However, their angiogenic and remodeling properties, as well as their role in ischemia-related endothelial leukocyte activation, need to be further elucidated. Herein, we investigated the effect of bone marrow–derived mesenchymal stromal cells (BM-MSCs) in a critically ischemic murine skin flap model. *Methods.* Groups received either 1×10^5, 5×10^5, or 1×10^6 BM-MSCs or cell-free conditioned medium (CM). Controls received sodium chloride. Intravital fluorescence microscopy was performed for morphological and quantitative assessment of micro-hemodynamic parameters over 12 days. *Results.* Tortuosity and diameter of conduit-arterioles were pronounced in the MSC groups ($P < 0.01$), whereas vasodilation was shifted to the end arteriolar level in the CM group ($P < 0.01$). These effects were accompanied by angiopoietin-2 expression. Functional capillary density and red blood cell velocity were enhanced in all treatment groups ($P < 0.01$). Although a significant reduction of rolling and sticking leukocytes was observed in the MSC groups with a reduction of diameter in postcapillary venules ($P < 0.01$), animals receiving CM exhibited a leukocyte-endothelium interaction similar to controls. This correlated with leukocyte common antigen expression in tissue sections ($P < 0.01$) and p38 mitogen-activated protein kinase expression from tissue samples. Cytokine analysis from BM-MSC culture medium revealed a 50% reduction of pro-inflammatory cytokines (interleukin [IL]-1β, IL-6, IL-12, tumor necrosis factor-α, interferon-γ) and chemokines (keratinocyte chemoattractant, granulocyte colony-stimulating factor) under hypoxic conditions. *Discussion.* We demonstrated positive effects of BM-MSCs on vascular regeneration and modulation of endothelial leukocyte adhesion in critical ischemic skin. The improvements after MSC application were dose-dependent and superior to the use of CM alone.

Key Words: *angiogenesis, arteriogenesis, conditioned medium, immunomodulation, mesenchymal stromal cells, paracrine function, vascular regeneration*

Introduction

In the past decade, mesenchymal stromal cells (MSCs) have been demonstrated to be a powerful cell population capable of promoting damaged tissue regeneration through modulation of both neovascularization and inflammation in critically ischemic tissue (1–3). MSC trophic and regenerative properties have opened the door for new cell-based therapeutic approaches to ischemic and inflammatory conditions.

Several studies have shown beneficial effects of adult bone marrow-derived MSCs (BM-MSCs) in animal models of localized ischemia, highlighting their ability to promote vascular regeneration of jeopardized vasculature. Stem cell treatment has been shown to augment arteriogenesis and angiogenesis in myocardial infarction as well as stroke (4–9). Initially, MSCs were assumed to incorporate physically in the regenerating tissue as endothelial- or tissue-specific cells (9–11). However, several recent studies have shown increasing evidence of a predominant modulatory role of MSCs in neovascularization through paracrine secretion of a broad

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spectrum of proangiogenic factors and chemokines. These factors orchestrate the attraction and migration of other cell types, influencing the repair mechanism in ischemic tissue (6,7,12–16). In this context, angiopoietin-2 (Ang-2) has been found to improve blood flow through arteriolar remodeling (17) of collateral vessels in critical ischemic diseases (18,19).

MSCs also exhibit anti-apoptotic and anti-inflammatory properties (1,3,20,21). On the basis of their immunomodulatory ability, they are promising candidates for treatment of autoimmune disorders such as rheumatoid arthritis and Sjögren syndrome (22,23), as well as allotransplantation-associated conditions such as graft-versus-host disease (1,24) and acute rejection (25,26). Indeed, adult stromal cells have been shown to constitutively release many immunomodulatory factors and influence leukocyte function and endothelial activation (27).

Therefore, various groups have begun to investigate the potential of MSCs to limit inflammatory responses and their sequelae in ischemic disorders (28,29), protecting jeopardized tissue from additional damage. However, the exact mechanisms underlying these effects have not been completely elucidated.

Initial clinical trials on the therapeutic use of MSCs in ischemia are underway (30–32), but an optimal cell dosage remains to be determined. To date, few studies have investigated a dose-response effect based on the amount of infused cells (33). Despite evidence that MSCs home to injury sites, the recruitment and homing of both injected and circulating or mobilized MSCs need to be further investigated (34,35). MSC therapy has shown some drawbacks in the past, such as entrapment in filtering organs (36–38) prohibiting homing to target tissues, scarce overall availability and invasive harvesting, which are just a few unsolved issues. Therefore, some authors have proposed the injection of cell-free conditioned medium (CM) containing a blend of chemokines and cytokines (39–41) as an alternative therapeutic option. However, the regenerative potential of CM still needs to be further addressed.

The aim of the current study was to compare the dose-response effects of intravenous single-time-point administration of different amounts of BM-MSCs to administration of CM alone on vascular regeneration and endothelial activation in critically ischemic murine skin.

Methods

Mesenchymal stem cell preparation

Six to 8-week-old female C57BL/6 mice (Charles River, Sulzfeld, Germany) served as stem cell donors. Femoral and tibial bones were flushed with sterile MACS Buffer (MACS Separation Buffer, Miltenyi Biotec, Bergisch Gladbach, Germany) using a 26-G needle as reported previously (13). The cell suspension was then centrifuged at 300*g* for 10 min. All isolation and separation steps were performed on ice under a laminar flow cabinet under sterile conditions. The pellets were resuspended in culture medium (α-minimal essential medium, 10% fetal calf serum 1% Pen/Strep solution, L-glutamine) and passed through 70-μm mesh. Cells were transferred to culture flasks and incubated until adherence (72 h). The medium was then removed, and cells were washed once with Dulbecco's phosphate-buffered saline (DPBS) to remove nonadherent cells, trypsinized and scratched off. The cells underwent lineage depletion (Lineage Cell Depletion Kit, Miltenyi Biotec) to remove hematopoietic cells (13). Depletion included CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C) and Ter-119 antibodies. All steps were performed according to the manufacturer's instructions.

The cells were characterized by fluorescence-activated cell sorting analysis (FACS). For this purpose, cells were cultured at high density ($2.5 \times 10^6/cm^2$) and incubated at 37°C with 5% CO_2 until adherence. The media was changed every 3 days. FACS was performed on BM-MSCs at passage 1 using a lineage-specific antibody kit (Mouse Multipotent Mesenchymal Stromal Cell 4-Color Flow Kit, FMC003, R&D Systems, Abingdon, United Kingdom) (26). The cells were immuno-labeled with direct labeled antibodies CD105 CFS, CD29 PE, Sca-1 APC, CD 45 PerCP and corresponding rat immunoglobulin G isotype control for each label according to the manufacturer's protocol. Cell fluorescence was the measured immediately with a FACS Flow Cytometer (FACSCanto Flow Cytometer, Becton Dickinson, and Company, Franklin Lakes, NJ, USA), and the data were analyzed with FlowJo Software v. 7.5 (Tree Star Inc., Ashland, OR, USA). At least 30,000 events were collected per measurement. All data were expressed as a percentage of positive cells as defined by flow cytometry. Multilineage differentiation (Mouse MSC Functional Identification Kit, R&D Systems) was demonstrated using oil-red-o staining for adipose differentiation and anti-osteopontin for the osteogenic lineage.

For systemic administration, the cells were isolated and depleted of hematopoietic cells as described above. After cell counting, the different cell numbers were re-suspended in 100 μL sterile 0.9% NaCl. Aliquots were kept on ice until tail-vein injection.

Conditioned medium

For the production of CM, BM-MSCs and murine embryonic fibroblasts (MEFs) were co-cultured to

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