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Dynamics of early stem cell recruitment in skin flaps subjected to ischemia reperfusion injury

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ABSTRACT

Objective: Bone marrow-derived stromal cell (BMSCs) therapy improves survival of skin flaps subject to ischemia/reperfusion (I/R) injury. However, very little is known about the trafficking and distribution of BMSCs in post-ischemic skin tissue following intravenous administration. The aim of this study was to assess the behavior of BMSCs in post-ischemic skin flaps and to compare the magnitude and kinetics of accumulation of BMSCs and leukocytes following I/R.

Methods: Cutaneous flaps perfused by the inferior epigastric vessels were created in C57Bl6 mice. The flaps were subjected to 3.5 h of ischemia followed by reperfusion. Wound healing and vascular perfusion were assessed in 3 groups of mice (sham, I/R, and I/R + BMSCs treatment) on days 3, 5, 7 and 14 post-reperfusion. The kinetics and magnitude of BMSCs and leukocyte recruitment were quantified in additional 2 groups (Sham and I/R) after I/R using intravital fluorescence microscopy at 2 and 4 h after the intravenous injection of fluorescently labeled BMSCs.

Results: Wound healing after I/R was significantly enhanced in skin flaps of mice treated with BMSCs, compared to controls. The rolling velocity of BMSCs was higher compared to leukocytes both in control mice (32.4 ± 3.7 μm/s vs 24.0 ± 2.2 μm/s, $p < 0.05$) and in I/R mice (34.6 ± 3.8 μm/s vs 20.2 ± 2.3 μm/s, $p < 0.005$). However, the rolling velocity of both cell populations was not altered by I/R. The firm adhesion and transendothelial migration of BMSCs did not differ from the values detected for leukocytes for both control and I/R mice.

Conclusions: The magnitude and kinetics of BMSCs recruitment in skin flaps subjected to I/R are not significantly different from the responses noted for leukocytes, suggesting that similar mechanisms may be involved in the recruitment of both cell populations following I/R.

1. Introduction

Reconstructive surgery of the head and neck in patients with burns, trauma and tumors frequently involves the use of various soft tissue flaps and grafts. Successful reconstruction relies on survival of the transplanted tissue. Soft tissue flaps and free grafts that have been subjected to ischemia/hypoxia are at risk for partial or total failure after I/R injury. I/R initially induces the release of pro-inflammatory mediators such as cytokines, chemokines and reactive oxygen species, elicits the recruitment of leukocytes, vascular leakage, and interstitial edema and ultimately leads to tissue necrosis [1,2]. Therefore, various therapeutic approaches including antioxidants [3], anti-inflammatory drugs [4], vasodilators [5], and pro-angiogenic growth factors [6,7] have recently been tested for prevention of I/R-induced tissue necrosis.

There is growing evidence that bone marrow derived stem cells (BMSCs) may improve ischemia related organ dysfunction and protect tissue from I/R injury [8,9]. The beneficial effects of BMSCs have been attributed to their immunomodulatory and anti-inflammatory actions [10]. It was once believed that BMSCs repair damaged tissues by robustly replacing the damaged cells because of their multipotent ability to differentiate into osteocytes, adipocytes, neural cells and vascular endothelial cells [11,12]. However, it is now generally appreciated that the paracrine and endocrine effects of BMSCs also contribute to their protective actions. The paracrine effects of BMSCs include 1) anti-apoptotic (VEGF, IGF-1 and bFGF), 2) supportive, including stimulation of mitosis, proliferation and differentiation of organ-intrinsic precursor cells (SCF, LIF and IL-6). 3) angiogenic (bFGF and VEGF),

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4) immunomodulatory (IL-10), 5) anti-scarring (IL-10 and bFGF), and 6) chemoattractant (MCP-1, MIP-1α/1β and IL-8) [13] actions. BMSCs also exhibit immunosuppressive properties against T cells [14], DCs [15], B cells [16], and NK cells [17]. BMSCs can also induce macrophages to assume an anti-inflammatory M2 phenotype, characterized by a high expression of IL-10 and low levels of IL-12 and TNF-α [18].

The process of leukocyte recruitment has been extensively described during an inflammatory response. Free flowing leukocytes are attracted by inflammatory chemokines (e.g., IL-8, MCP-1 and SDF-1), attach to activated endothelial cells, and emigrate from the circulation into the area of tissue injury. This multi-step process, includes selectin-dependent tethering and rolling, activation, integrin-dependent adhesion, strengthening and spreading, intravascular crawling, paracellular and transcellular transmigration [19]. Relative to leukocytes, the existing knowledge regarding how transplanted BMSCs are recruitment by the injured tissue is limited. Since stem cells and leukocytes are from the same lineage, the adhesive and transmigrated mechanisms of BMSCs is postulated to occur in a manner similar to that described for leukocytes. BMSCs recruitment is thought to involve their migration along an SDF-1 gradient and their adhesion to different molecules produced and expressed by hypoxic and injured tissue [20].

Recent studies have demonstrated that HSCs express a repertoire of surface adhesion molecules that is similar to mature leukocytes, which includes CD29 (β1) and CD18 (β2) integrins binding to their endothelial counter-receptors VCAM-1 and ICAM-1, respectively [21]. A critical role for the integrin CD49d/VCAM-1 pathway in mediating stem cells recruitment to ischemically injured murine kidney has been demonstrated [22]. BMSCs are detected in ischemic kidney tissue within the first hour after intravenous administration [23]. Other studies have shown that BMSCs emigrate across TNF-α-activated endothelium and become partially integrated in the endothelial layer in inflamed tissue [24]. However, in another study it was demonstrated that MSCs administered intravenously in an animal model of liver I/R are short-lived and do not migrate into the target organ [25]. Therefore, despite the existence of many reports that describe an improved survival of post-ischemic tissues, including skin flaps [26,27], following treatment with stem cells, relatively little is known about how these cells are recruited into tissues following I/R [28]. Indeed, no attempt has previously been made to compare the recruitment responses of BMSCs with leukocytes in the same tissue following I/R. Hence, the objective of this study was to compare the kinetics and magnitude of BMSCs recruitment in a skin flap preparation subjected to I/R to the recruitment response observed for leukocytes.

2. Materials and methods

2.1. Animals

All mice were used on a C57Bl/6 J background (male; 12 weeks old; Jackson Laboratories, Bar Harbor, ME). Animals were housed in a barrier facility and maintained on a normal diet ad libitum. Experimental procedures were in compliance with the guidelines of both the Louisiana State University Health Institutional Animal Care and Use Committee and the National Institutes of Health. All efforts were made to minimize animal distress and the number of animals used.

2.2. Model of cutaneous flap ischemia and reperfusion

C57Bl/6 J mice were anesthetized with isoflurane gas. After hair removal, a 1 × 2 cm cutaneous flap perfused by the inferior epigastric vessels were raised on the abdomen. These dimensions were selected in order to keep the flap unilateral to one side of the abdomen and to ensure no collateral flow from more superiorly based vessels. The pedicle was clamped (producing ischemia) for 3.5 h. The body temperature was maintained and the flap tissue was kept moist. Following release of the clamp, and 1 h of reperfusion, 3×10^6 BMSCs in 150 μl PBS were administered via the femoral vein for the investigation of wound healing or intravital fluorescence microscopy.

2.3. BMSC isolation, culture and labelling

The bone marrow-derived stem cells were isolated from H-2Kb-tsA58 mice expressing the temperature-sensitive SV40 large-T antigen (large T; CBA/ca X C57Bl/10 hybrid; Charles River Laboratories, Wilmington, MA). Briefly, fresh complete bone marrow were harvested aseptically from tibias and femurs and cultured in Dulbecco's Modified Eagle Medium (DMEM; Fisher Scientific; US) supplemented with 10% fetal bone serum (FBS; Gemini Bio-Products; US) and 1% anti-biotic/anti-mycotic (Fisher Scientific; US). After 3 days of incubation, non-adherent cells were removed. BMSCs were harvested using 0.25% trypsin EDTA dissociation solution. The cells were then labeled by incubation with 20 μM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies Corporation; US) for 10 min, followed by wash and centrifugation. The cells were filtered through a 40 μm cell strainer and resuspended in PBS (pH 7.4). 3×10^6 cells were injected intravenously after 3.5 h of ischemia and 1 h of reperfusion into mice within 10 min prior to obtaining estimates of cell trafficking via intravital videomicroscopy.

2.4. Intravital fluorescence microscopy

Following injection (i.v.) of CFSE-labeled BMSC and 30 μl 0.02% rhodamine–6 G (Sigma, US), the free skin flap was covered with a glass coverslip. The space between the glass and skin was filled with 0.9% saline. The mouse was placed on the stage of an upright fluorescent microscope (BX51WI; Olympus, Japan), maintaining body temperature at 35–36 °C. Visualization of individual skin flap microvessels and cells was achieved using a 20 × water immersion objective lens (UMPlanFL N 20 × /0.50; Olympus). A color video camera (3CCD, Sony, Japan) projected the images onto a monitor (Trinitron; Sony). The images were recorded with Pinnacle Studio 17 software. The diameters and length of the selected microvessels were measured using image analysis software (Image J; NIH, US) on a personal computer (Dell; US).

2.5. BMSC rolling, adhesion and transmigration analysis

All experimental images were recorded using the same dimensions (1456 × 2592 pixels) and analyzed off-line. 8–10 vessels (diameter 20–40 μm) of the *panniculus carnosus* skeletal muscle were analyzed in each skin flap/animal. Rolling cells were defined as cells that transiently interact with the vessel wall. The flux fraction of rolling cells was calculated for each recording by dividing the number of rolling cells by the total number of cells passing the vessel. Adherent cells were defined as cells that remain stationary on the blood vessel wall for ≥ 30 s. Percentage of adherent cells was calculated for each recording by dividing the number of adherent cells by the total number of cells passing the vessel plus adhesion per minute. Transmigrated cells were defined as cells that were outside the boundary of the blood vessel and within the adjacent interstitial compartment.