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Paracrine effects of mesenchymal stem cells enhance vascular regeneration in ischemic murine skin

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ABSTRACT

New theories on the regeneration of ischemic vasculature have emerged indicating a pivotal role of adult stem cells. The aim of this study was to investigate homing and hemodynamic effects of circulating bone marrow-derived mesenchymal stem cells (MSCs) in a critically ischemic murine skin flap model.
Bone marrow-derived mesenchymal stem cells ($Lin^{-}CD105^{+}$) were harvested from GFP^{+}-donor mice and transferred to wildtype C57BL/6 mice. Animals receiving GFP^{+}-fibroblasts served as a control group. Laser scanning confocal microscopy and intravital fluorescence microscopy were used for morphological analysis, monitoring and quantitative assessment of the stem cell homing and microhemodynamics over two weeks. Immunohistochemical staining was performed for GFP, eNOS, iNOS, VEGF. Tissue viability was analyzed by TUNEL-assay.
We were able to visualize perivascular homing of MSCs in vivo. After 4 days, MSCs aligned along the vascular wall without undergoing endothelial or smooth muscle cell differentiation during the observation period. The gradual increase in arterial vascular resistance observed in the control group was abolished after MSC administration ($P<0.01$). At capillary level, a strong angiogenic response was found from day 7 onwards. Functional capillary density was raised in the MSC group to 197% compared to 132% in the control group ($P<0.01$). Paracrine expression of VEGF and iNOS, but not eNOS could be shown in the MSC group but not in the controls.
In conclusion, we demonstrated that circulating bone marrow-derived MSCs home to perivascular sites in critically ischemic tissue, exhibits paracrine function and augment microhemodynamics. These effects were mediated through arteriogenesis and angiogenesis, which contributed to vascular regeneration.
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Introduction

The interruption of blood supply leads to ischemia and hypoxia in the corresponding vascular territory with consecutive functional loss and cell death. Previous attempts to tackle critical ischemia have focused on improving both microcirculation and oxygenation in the jeopardized tissue with pharmacological measures affecting the architecture and tone of the vascular wall, blood rheology and coagulability, inflammation and endothelial function (Banz et al., 2005; Cannon, 2006; Dayspring, 2001; Dawson et al., 2002; Sudano et al., 2006). During the last few years, a new approach has emerged which consists of promoting the regenerative potential of the vasculature to compensate for the diminished supply of blood and oxygen. Such neovascularization includes the formation of new vessels (angiogenesis) and the remodeling (arteriogenesis) of pre-existing vascular structure (Carmeliet, 2000; Cao et al., 2005; Heil and Schaper, 2004; Heil et al., 2006; Jain and Duda, 2003). Recent observations have suggested that adult mesenchymal stem cells (MSCs) may play a pivotal role in both of these mechanisms after ischemic tissue damage and wound healing (Park et al., 2004, 2007). By their embryological origin, MSCs are a putative cellular component of these processes (Loscalzo, 2004; Quesenberry et al., 2004). Vascular regeneration follows a specific time pattern, which depends on the preexisting vascular system, the type of tissue and the level of ischemia. The expression of ephrins and their receptors in the early course suggests an involvement of stem cells in terms of vasculogenesis (Erber et al., 2006; Heil et al., 2006; Jain, 2003).

MSCs are mainly derived from bone marrow but can also be found in stromal tissues like muscle and fat in adult organisms. A small fraction is continuously released into circulation (Aicher et al., 2007; Asahara et al., 1997; Shantsila et al., 2007). These cells are non-hematopoietic and

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express a subset of antigens like CD105, CD34, CD133, and Stro-1 (De Ugarte et al., 2003b). Stem cells of higher differentiation, e.g. endothelial progenitor cells and smooth muscle-like progenitor cells, are thought to be part of this fraction and could be relevant in vascular regeneration (Asahara et al., 1997). Circulating bone marrow-derived MSCs have also been identified to share phenotype with monocyte sub-populations, which would be relevant for migration abilities (Zentilin et al., 2006). Recently, the paracrine function of stem cells has been demonstrated in tumor angiogenesis (Fang and Salven, 2011; Wickersheim et al., 2009).

In this study, the primary goal was to visualize, quantify and monitor the homing of bone marrow-derived circulating MSCs and their effect on vascular regeneration in murine flap skin exposed to critical ischemia.

Methods

Study design and animals

The experiments were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals and with the approval of the local Animal Ethics Committee. Three groups were studied. The first group was a control group receiving an intravenous post-operative tail vein injection of 100 μl sodium chloride (NaCl 0.9%). The second group received an intravenous injection of 2×10^5 fibroblasts in 100 μl sodium chloride. This group was designed to serve as a control group receiving fibroblasts as cellular control. The third group was the focus group. These animals received 2×10^5 freshly isolated Lin^-CD105^+ bone marrow-derived mesenchymal stem cells in 100 μl sodium chloride via tail vein injection post-operatively on the first day after flap surgery.

Twenty-one C57BL/6 female mice between 10 and 12 weeks of age underwent surgery to visualize, monitor and quantify the microvascular changes over 14 days in each animal. Fifteen C57BL/6 mice served for tissue sampling on day 7 (5 per group), four animals served for laser-scanning confocal microscopy (LSCM) investigations after receiving MSC transplantation and twenty GFP^+ animals served as stem cell and fibroblast donors. The GFP^+ mice were bred on a C57BL/6 background (C57BL/6-Tg(UBC-GFP)30Scha/J, Jackson Laboratories, Bar Harbor, USA).

Wound infection and technical complications such as wound dehiscence and insufficient optical clarity for intravital fluorescence microscopy (IFM) due to granulation tissue were defined as exclusion criteria.

Surgery

A murine dorsal skin flap model was used as described previously (Schweizer et al., 2011). In brief, systemic anesthesia was induced and an island flap measuring 25 × 13 mm was raised on one side of the dorsal midline, pedicled on the lateral thoracic vessels. The flap included the vascular territories of the lateral thoracic artery in the proximal part and the superficial circumflex iliac artery in the distal part. During dissection, the superficial circumflex iliac vessels were transected, thus rendering the corresponding vascular territory in the distal part of the flap critically ischemic. The flap consisted of skin, subcutaneous tissue, and a thin layer of panniculus carnosus muscle. During surgery, the flap was irrigated with 0.9% NaCl to prevent it from drying out. The flap was sutured back into its original position. A window was created on the opposite side, and a skin fold chamber was mounted to enable visualization of the flap from under the surface for repetitive observation by intravital microscopy.

Protocol

For all manipulations, the animals were anesthetized with an intraperitoneal injection of medetomidine 500 μg/kg body weight (BW), climazolam 5 mg/kg BW, and fentanyl 50 μg/kg BW according to established protocols (Schlosser et al., 2010; Schweizer et al., 2011; Vihanto et al., 2005). Reversion was induced by antidote injection with atipamezol 1.25 mg/kg BW (Antisedan, Pfizer, Switzerland), sarmazenil 0.5 mg/kg BW (Sarmasol, Graeub, Switzerland) and naloxon 0.6 mg/kg BW (Naloxon, Orpha, Switzerland) after the manipulations were completed. The anesthetized animals were placed on a heating pad in a prone position, and the room temperature was set at 28 °C to keep their skin temperature constant at 32 °C, which was verified with a microthermometer placed on the abdominal skin. The back skin was shaved and depilated. Surgery was performed with the aid of an operating microscope at ×10 magnification (Wild, Heerbrugg, Switzerland). Post-operatively, animals received analgesic treatment with metamizole (0.25 mg/g BW, Novalgin, Sanofi-Aventis, Switzerland) and 0.l ml glucose 5% for volume replacement purposes due to the large wound size.

Repeated measurements were performed under anesthesia on days 1, 4, 7, 10 and 14 post-operatively by the use of IFM.

Microhemodynamic measurements

Investigations were performed with an epiluminescence intravital microscope (Zeiss AxioplanI, Zeiss, Jena, Germany). Microscopic images were captured by a television camera (intensified CCD camera; Kappa Messtechnik, Gleichen, Germany), displayed on a television screen (Trinitron PVM-1454; Sony, Tokyo, Japan), and recorded on video (50 Hz; Panasonic AG-7350-SVHS, Tokyo, Japan) for subsequent off-line analysis as reported earlier (Contaldo et al., 2005; Plock et al., 2005). The preparation was observed visually with a ×40 water immersion objective with a numerical aperture of 0.75, which resulted in a total optical magnification of ×909 on the video monitor, where 1 pixel corresponded to 264 nm of tissue. The microvessels were classified according to physiological and anatomical features (Erni et al., 1999). Capillaries were chosen for evaluation according to their optical clarity. The intraluminal microvascular diameters and the relative length were measured visually on the television screen in μm with the use of 2% fluorescein isothiocyanate-labeled dextran (FITC dextran, 150 kDa; Sigma, Buchs, Switzerland). Relative length was defined as length of the vessel correlated to the distance between two branching points in A0 arterioles. Elongation could be observed in terms of corkscrew formation due to arteriogenesis. Angiogenesis was quantified in terms of functional capillary density (FCD), which was defined as the length of red blood cell-perfused capillaries per observation area (cm/cm^2). To determine the qualitative perfusion of capillaries, the capillary perfusion index (CI) was calculated as a function of functional capillary density (FCD) and red blood cell velocity (RBCV) as previously established (Plock et al., 2005; Plock et al., 2007): $CI = FCDxRBCV$. This parameter provides a quantitative reflection of volumetric capillary blood flow through calculation of the amount of erythrocytes passing through the capillary system per unit of time.

The data were obtained from a computer-assisted image analysis system (CapImage; Zeintl Software, Heidelberg, Germany).

The approximate arteriolar vascular resistance was calculated to determine the influence of lengthening (length: l) and dilation (radius: r) of the A0 artery on perfusion pressure (R) according to the Hagen-Poiseuille equation: $\Delta R = \Delta l/\Delta r^4$.

Isolation and separation of mesenchymal stem cells

Eight week-old female GFP^+ mice were sacrificed. Femoral and tibial bones were flushed with sterile MACS Buffer (MACS Separation Buffer, Miltenyi Biotec, Germany) using a 26 G needle. The cell suspension was then centrifuged at 300 g for 10 min. All isolation and separation steps were performed on ice.

After re-suspending the pellet in MACS buffer cells underwent lineage depletion (Lineage Cell Depletion Kit, Miltenyi Biotec, Germany) to remove hematopoietic cells. Lineage depletion included the following

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