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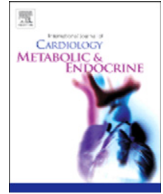

Aging impairs ischemia-induced neovascularization by attenuating the mobilization of bone marrow-derived angiogenic cells

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ABSTRACT

Background: Aging is associated with impaired ischemia-induced neovascularization. However, the effects of aging on bone marrow-derived angiogenic cell (BMDAC)-mediated vasculogenesis and on angiogenesis at the ischemic sites remain incompletely understood.

Methods and results: Two- and 24-month old male C57Bl/6J mice were subjected to hindlimb ischemia. The levels of Sca1+/CXCR4+ BMDACs were determined post-ischemia by flow cytometry. In young mice, ischemia increased Sca1+/CXCR4+ BMDAC levels in the bone marrow and spleen at day 3 ($p < 0.001$) and in the circulating blood at day 7 ($p < 0.01$) post-ischemia. However, ischemia-induced elevation of progenitor cells was attenuated in the bone marrow, spleen and blood of old mice despite a preserved HIF-1α-mediated angiogenic response in the ischemic tissues. Irradiated young recipient mice engrafted with old bone marrow displayed reduced levels of Sca1+/CXCR4+ BMDACs in the bone marrow and circulating blood post-ischemia compared to recipients with young bone marrow. *Ex vivo* cultured BMDACs from old mice exhibited reduced SDF-1-stimulated migration ($p < 0.01$) and a decrease in JAK-2 and AKT activation. However, the intrinsic angiogenic function of BMDACs, including VEGF secretion and promotion of endothelial cell tubule formation, was preserved with aging. Furthermore, facilitated mobilization of old bone marrow-derived mononuclear cells to the ischemic hindlimb by intramuscular injection enhanced ischemia-induced neovascularization in old mice *in vivo* ($p < 0.001$).

Conclusions: The age-related impairment in ischemia-induced neovascularization is largely attributable to a marked attenuation of BMDAC mobilization with a preservation of intrinsic angiogenic function with age.

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1. Introduction

Aging is an unavoidable biological deteriorative process that is associated with a decline in adaptive responses toward different stressors, including ischemia. Aging alone is a major risk factor for coronary and peripheral artery disease and is also associated with increased incidence of other cardiovascular diseases [1]. The majority of cardiovascular disease-related deaths are in elderly individuals aged 75 years and older. Cardiovascular disease in the elderly is hallmarked by impairment in key vascular repair and regeneration processes including ischemia-induced neovascularization [2–7]. With an increasingly aged global population, there is a need to better understand the mechanisms of age-related impairment in vascular repair in an attempt to provide better therapies for elderly patients with vascular diseases.

Postnatal neovascularization is a key mechanism for cardiovascular repair and regeneration following ischemia that relies on two interrelated processes: vasculogenesis and angiogenesis. Vasculogenesis involves the proliferation and mobilization of angiogenic progenitor cells from the bone marrow into the circulating blood [8–10]. Bone marrow-derived angiogenic progenitor cells (BMDACs) consist of heterogeneous cell populations which have been previously described as endothelial progenitor cells, circulating angiogenic cells or proangiogenic myeloid cells [11–13]. These progenitor cells with divergent angiogenic capabilities have been shown to promote blood perfusion at the ischemic sites by incorporating into newly formed blood vessels and/or promoting vascular growth by producing angiogenic cytokines [14–15]. Angiogenesis is defined as the formation of new blood vessels from the preexisting ones. Ischemia-induced upregulation of a transcription factor hypoxia-inducible factor-1α (HIF-1α) mediates the expression of angiogenesis-

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related genes, such as vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1) [16–18]. HIF-1α mediated upregulation of SDF-1 facilitates the mobilization of progenitor cells into the circulation through the binding to its receptor, C-X-C receptor 4 (CXCR4) [19–21]. Upon binding of SDF-1 to its receptor CXCR4, janus kinase (JAK) and phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathways are activated to induce cell mobilization [22–24].

To date, the mechanisms underpinning the age-related decline in ischemia-induced neovascularization remain incompletely understood. The relative contribution of changes in the regenerative capacity of circulating progenitor cells ("the seed") *versus* changes in the pro-regenerative environment in ischemic tissues ("the soil") to the age-related impairment in neovascularization remains debated. In this study, we investigated the effect of aging on ischemia-induced neovascularization by using a murine hindlimb ischemia model involving young and aged mice. We also examined the differential effects of aging on the vasculogenic and angiogenic potential of BMDACs *in vivo* and *in vitro*.

2. Methods & materials

2.1. Mouse hindlimb ischemia (HLI) model

All animal studies were conducted with ethical approval from the Sydney Local Health District Animal Ethics Committee (#2011-001A). Two-month old male C57Bl/6J mice were purchased from Australian BioResources and 24-month old male C57Bl/6J mice were purchased from the National Institute of Aging (Bethesda, MD). Mice were housed in a temperature-controlled environment with a 12 hour light and 12 hour dark cycle. Under inhalation of methoxyflurane, mice underwent unilateral hindlimb ischemia (HLI) by femoral artery ligation and excision [25]. A sham procedure was performed on the contralateral limb. Adequate anesthesia was confirmed throughout surgery by testing the pedal reflex. Carprofen (5 mg/kg) was used for analgesia, administered subcutaneously following surgery. Mice were monitored daily to determine the need of carprofen administration. Laser Doppler perfusion imaging (LDPI) was performed prior to surgery, post-surgery and at days 3, 5, 7, 10 and 14 post-ischemia (moorLDI2-IR, Moor Instruments, UK). Blood flow was displayed as a heat map, in which dark blue indicated minimal and red indicated maximum blood flow. Blood perfusion ratio was expressed as the ratio of blood flow in the ischemic *vs.* the non-ischemic limb. Mice were euthanized at the indicated days for experimental purposes.

2.2. Mononuclear cell (MNC) isolation

Upon sacrifice, femur and tibia were flushed with phosphate-buffered saline (PBS) to collect bone marrow. Spleens were homogenized in PBS through a 70 μm nylon filter. Mononuclear cells (MNCs) from the bone marrow, blood and spleen were isolated using Lympholytes®-M Cell Separation Media (Cedarlane, Burlington, Ontario). Total counts of mononuclear cells are included in Supplementary Table 1 in the online version at http://dx.doi.org/10.1016/j.ijcme.2016.05.005. Cells were centrifuged at 740 ×g for 30 min. Buffy coats were collected and washed twice with PBS + 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). Cells were resuspended in EGM-2 medium (Lonza, Basel, Switzerland) for *ex vivo* culture and flow cytometry analysis.

2.3. Bone marrow transplantation

Young recipient mice were irradiated once at 1000 rad dose prior to bone marrow transplantation. Donor bone marrow cells (BMCs) were flushed from the femur and tibia of young and old mice with RPMI medium (Sigma). Cell suspensions were filtered with a 70 μm nylon mesh and resuspended in the appropriate volume of RPMI for intravenous injection (1×10^6 cells in 200 μl RPMI injected per recipient). Following transplantation, recipient mice were kept in a sterile environment for 6 weeks prior to any experimental surgery.

2.4. Intramuscular injection of MNCs

Bone marrow MNCs isolated from old donor mice were intramuscularly injected in PBS into old mice immediately after HLI surgery (1×10^6 MNCs per recipient). PBS alone was used in control animals. Blood flow recovery was monitored for 14 days post-ischemia using LDPI.

2.5. Immunohistochemistry

Upon sacrifice, thigh adductor muscles were collected and embedded in Tissue-Tek OCT compound (Finetek, New Taipei City, Taiwan). Embedded tissue was snap-frozen in liquid nitrogen and stored at −80 °C until use. Tissue cryosections (7 μm thick) were fixed in cold 3.7% paraformaldehyde for 10 min and rinsed with PBS. Capillary density, arteriolar density and vessel sizes were examined by immunostaining with rat monoclonal anti-laminin (Abcam, Cambridge, UK), rat monoclonal anti-CD31 conjugated to phycoerythrin (Abcam) and mouse monoclonal α-smooth muscle actin conjugated to fluorescein isothiocyanate (FITC) (Sigma). Goat anti-rat IgG conjugated to Alexafluor 350 (Life Technologies, Grand Island, NY) was used as a secondary antibody. The capillary density (positive for CD31+) and arteriolar density (positive for α-smooth muscle actin) and vessel luminal sizes were normalized to the numbers of myocytes.

2.6. Immunofluorescence terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining

Apoptotic cells were determined by a DeadEND Fluorometric TUNEL system (Promega), according to the manufacturer's protocol. Briefly, OCT-embedded tissues were fixed with 4% paraformaldehyde for 15 min and washed twice with PBS for 5 min. After incubation in permeabilization solution for 10 min and washing with PBS twice, tissue samples were incubated with TUNEL reaction mixture at 37 °C in humid conditions for 1 h and encapsulated by mounting medium Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). TUNEL-positive and DAPI-positive cells were counted in ten randomly selected microscopic fields. The percentages of apoptotic cells were expressed as TUNEL-positive cells per total number of DAPI-positive cells.

2.7. Flow cytometry analysis

A subset of progenitor cells from bone marrow-derived angiogenic cell (BMDAC) populations was identified as Sca1+/CXCR4+ BMDACs by flow cytometry using the hematopoietic stem cell marker Sca1 and the SDF-1 receptor CXCR4, as previously described [26,27]. Isolated bone marrow, spleen and blood MNCs were incubated with FcR blocking solution (Miltenyi Biotec, Bergisch Gladbach, Cologne) according to the manufacturer's protocol at 4 °C for 15 min. Cells were then labelled with anti-mouse Sca1-V450 and anti-mouse CXCR4-APC (BD Bioscience, San Jose, CA) at 4 °C for 30 min. Cells were then washed twice with PBS + 10% FBS and resuspended in PBS + 2% FBS for flow cytometric analysis of Sca1+/CXCR4+ BMDACs (BDFACSVerse, Becton Dickinson, Franklin Lakes, NJ).

2.8. Ex vivo culture of bone marrow-derived angiogenic cells (BMDACs)

BMDACs were cultured as previously described [28]. Briefly, isolated bone marrow MNCs were plated on fibronectin-coated 24-well plates at 2×10^6 cell density per well in EGM-2 + 10% FBS medium. Cells were fed with fresh medium on alternate days. On day 5, cells were washed with PBS and labelled with 3 μg/ml acetylated low density lipoprotein

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