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Adipose-derived stromal vascular fraction cells isolated from old animals exhibit reduced capacity to support the formation of microvascular networks

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ABSTRACT

Adipose-derived regenerative and stem cells, defined collectively as the stromal vascular fraction (SVF), support the formation of neovascular networks at the site of implantation. The effect of advancing age on SVF cell population effectiveness towards stimulated neovascularization was evaluated.

Methods: SVF was enzymatically isolated from adipose of young (ySVF, 4 months) or old (oSVF, 24 months) Fisher-344 rats, combined with type I collagen and polymerized. Encapsulated SVF was implanted subcutaneously into young Rag1 mice for two or four weeks. Angiogenic function of age-dependent SVF was also extensively evaluated in vitro using standard assays.

Results: In vitro studies indicated no difference in angiogenic function between ySVF and oSVF (viability, proliferation, migration, and tube-formation). At two weeks post-implantation, there was no age-related difference in percent apoptosis in explanted constructs. By four weeks post-implantation, oSVF implants displayed 36% less total vessels/mm^2, 43% less perfused vessels/mm^2, and exhibited greater percent apoptosis compared to ySVF ($n \geq 12$). Blocking thrombospondin-1 (Thbs-1), a protein found to be highly expressed in oSVF but not ySVF, increased the percent of perfused vascular volume and vessel diameters in oSVF constructs after two weeks compared to oSVF implants treated with control antibody.

Conclusions: Advancing donor age reduces the potential of adipose-derived SVF to derive a mature microcirculation, but does not hinder initial angiogenesis. However, modulation of Thbs-1 may improve this outcome. This data suggests that greater pruning, dysfunctional structural adaptation and/or poor maturation with initiation of blood flow may occur in oSVF.

1. Introduction

Peripheral vascular disease (PVD) and myocardial ischemia persist in part due to the body's inability to spontaneously revascularize through the processes of angiogenesis and vasculogenesis. Therapeutic attempts to treat ischemia with angiogenic factors have been largely ineffective (Collinson and Donnelly, 2004). Cell-based therapies have been initiated with the hypothesis that cells may provide a more effective mixture of secreted factors to stimulate revascularization. Previously our laboratory and others have demonstrated that freshly isolated adipose-derived regenerative and stem cells can induce new vascular network formation through the reassembly of endothelial cells at the site of implantation (Koh et al., 2011; Nunes et al., 2013). The formation of new patent vessels has been shown to involve both de-novo vessel formation from existing vessels (angiogenesis) and the formation of new vessels through self-assembly of transplanted cells

Abbreviations: 3D, three dimensional; ADSC, adipose-derived stromal cells; Angpt2, angiopoietin-2; Angptl4, angiopoietin-like 4; α-Thbs-1, anti-thrombospondin-1; BMSC, bone-marrow derived mesenchymal stromal cells; BSA–PBS, bovine serum albumin in phosphate buffered saline; CM, conditioned media; CXCL5, chemokine (CXC motif) ligand 5; DAPI, 4′,6-diamidino-2-phenylindole; Dex, dextran–rhodamine; GS-1, *Griffonia simplicifolia* I; Hgf, hepatocyte growth factor; IHC, immunohistochemistry; MSC, mesenchymal stem cell; oSVF, SVF from old donor; oSVF + IgG, oSVF plus control IgG antibody; oSVF + α-Thbs-1, oSVF plus α-Thbs-1 antibody; P1, passage 1; PDGFβ, platelet-derived growth factor beta polypeptide; PVD, peripheral vascular disease; SVF, stromal vascular fraction; Tgfβ1, transforming growth factor beta 1; Thbs-1, thrombospondin-1; Tie1, tyrosine kinase with immunoglobin-like and EGF-like domains 1; Tnf, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; ySVF, SVF from young donor; VEGF, vascular endothelial growth factor; VVIF, vascular volume index fraction.

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(vasculogenesis). Implanted adipose-derived cells progress through distinct phases of neovascularization to eventually form a complete vascular network: vasculogenesis/angiogenesis, vascular remodeling and pruning, and finally, vascular maturation (LeBlanc et al., 2012; Nunes et al., 2010).

The majority of studies on neovascularization potential of cells of mesenchymal origin have used bone marrow-derived sources (BMSCs). More recently, adipose-derived stromal cells (ADSCs) have been shown to possess similar pro-angiogenic capabilities. ADSCs can form vascular networks by direct interactions with endothelial cells through cell incorporation (Merfeld-Clauss et al., 2010), but also through extensive paracrine mechanisms to modulate the process of angiogenesis (Rehman et al., 2004; Yang et al., 2013). Comparatively, human ADSCs have been shown to exhibit superior therapeutic potential when compared to human BMSCs in a mouse hindlimb ischemia model (Kim et al., 2007). Even though clinical presentations of ischemia so often occur in states of advanced age, there have been surprisingly few studies utilizing adipose-derived cells from older individuals to determine age-related potential for vascular therapy. The neovascularization potential of the adipose-derived regenerative and stem cell population from aged donors may be diminished.

Advanced age has been reported to be associated with poor wound healing responses suggesting that an age-related defect exists in the cell-dependent mechanisms that regulate neovascularization. Whether the age of the donor renders these cells ineffective in overall neovascularization potential or a deficit in one of the phase components remains unknown, but is important to ascertain if an autologous cell-based approach is to be utilized for patients with vascular ischemia. Numerous studies have suggested that adipose tissue from older humans and animals demonstrates cellular deficiencies, such as lower proliferation rates and greater susceptibility to apoptosis (Schipper et al., 2008). The general consensus is mesenchymal stem cells (MSCs) and progenitor cells lose proliferative and differentiation capacity with advancing age (Stolzing et al., 2008), but this loss of cell function hasn't been investigated in the heterogeneous adipose-derived stromal vascular fraction (SVF) cell populations.

The SVF represents a heterogeneous cell population that resides within adipose tissue and is comprised of endothelial cells, smooth muscle cells, macrophages, blood cells and mesenchymal stem cells (Zimmerlin et al., 2010). Adipose-derived cells have recently become the focus of numerous efforts designed to mitigate ischemia and heart failure in animal models (Mazo et al., 2008; Miyahara et al., 2006; Wang et al., 2009; Zhang et al., 2007) and clinical applications (Gir et al., 2012). Adipose tissue represents an attractive cell source for regenerative medicine due to the ease of isolation, abundance, and the ability of some cells to differentiate into various mesodermal cell lineages (Lin et al., 2008). The purpose of this study was to directly assess the age-related neovascularization potential of isolated SVF cells in an in vivo model with further in vitro characterization. Our overall hypothesis was that SVF isolated from aged rats (oSVF) would display decreased vasculogenic function in vivo compared to cells from young rats (ySVF), and that this would be decreased due to impaired capillary-like tube formation. Contrary to our hypothesis, we provide evidence here that the vasculogenic potential of SVF appears to be maintained with advancing age, but network maturation is impaired in oSVF in vivo. We believe greater pruning and/or poor adaptation to the initiation of perfusion and inosculation results in fewer cells and vessels maintained when oSVF cells are used.

2. Materials and methods

All animal surgeries were performed in accordance with protocols approved by the University of Louisville Institutional Animal Care and Use Committee and the NIH *Guide for the Care and Use of Laboratory Animals* (9th ed., 2011).

2.1. SVF Isolation

Young (4 months) and old (24 months) male and female Fischer-344 rats (Harlan Laboratories, Indianapolis, IN, USA and National Institute on Aging, Bethesda, MA, USA, respectively) were anesthetized (40–80 mg/kg ketamine and 5–10 mg/kg xylazine), epididymal or ovarian fat was excised, and the rat was euthanized by removal of the heart. The fat was washed in 0.1% bovine serum albumin in phosphate-buffered saline (BSA–PBS), finely minced, and enzymatically digested with 2 mg/mL type I collagenase (type I collagenase, Worthington Biochemical Company, NJ) for 40 min at 37 °C. After digestion, the tissue-collagenase mixture was centrifuged, buoyant adipocytes were removed, and the top two layers of the cell pellet were retrieved to obtain the SVF.

2.2. Construct formation and implant

As previously described, young rat SVF (ySVF) or old rat SVF (oSVF) cells were used immediately after isolation for construct implants by combining with BD Biosciences rat-tail type I collagen (3.0×10^6 cells/mL) and 4× DMEM to form three-dimensional (3D) constructs (Nunes et al., 2011). Briefly, two constructs per mouse were subcutaneously implanted into the dorsal flank of 6 week old immunodeficient Rag1 mice (B6;129S7-Rag1^{tm1Mom}/J, Jackson Laboratories, Bar Harbor, MA, USA). At four weeks post-implant, the mice were perfused with 250 μL of 2 mg/mL dextran–rhodamine (Dex+, 2,000,000 MW, Invitrogen) for 15 min before construct explant to delineate perfused vessels. One construct from each mouse was designated for confocal microscopy imaging and the other for immunohistochemistry (IHC). Following explant, IHC constructs were fixed in paraformaldehyde while those for confocal imaging were fixed, permeabilized, and stained with 0.1 mg/mL fluorescein-conjugated *Griffonia simplicifolia* I (GS-1) lectin overnight (Vector Labs) to label all vascular elements, and confocal laser microscopy was performed (Olympus model BX61WI; Olympus America Inc, Central Valley, PA).

2.3. Vessel perfusion and vessel density analysis

To estimate the vascular volume index fraction (VVIF), individual slices of confocal image stacks obtained at 20× magnification were processed as previously reported (Nunes et al., 2010). The percent perfusion of vasculature is calculated by dividing the volume of perfused vessels by the entire vessel volume ($Dex+_{VVIF}$ / $GS\text{-}1+_{VVIF}$), averaged from three image stacks throughout each construct. The absolute number of Dex+ and GS-1+ vessels was calculated manually by using the counting tool in Adobe® Photoshop (Adobe Systems Incorporated, San Jose, CA). Results are presented in multiple aspects: as the number of GS-1+ and Dex+ vessels per mm^2, percent perfused vessel volume relative to total, and vessel diameter distribution for ySVF or oSVF constructs. Vessel diameter distribution was calculated by measuring diameters of all vessels present in a 3D construct (ySVF or oSVF) and binning them in 5 μm increments.

2.4. LIVE/DEAD assay

The LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies) was used per manufacturer's instructions to determine viability after cell isolation. 3 images/sample were taken with a fluorescent microscope (Olympus model IX51; Olympus America Inc, Central Valley, PA) and averaged to determine percent viability ([# live cells / # dead cells] * 100).

2.5. RNA and RT-qPCR for angiogenic markers

The RT2 Profiler PCR Array for Rat Growth Factors (Qiagen) was used to evaluate 84 genes related to angiogenic growth factors. RNA was