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Age-related focal loss of contractile vascular smooth muscle cells in retinal arterioles is accelerated by caveolin-1 deficiency

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ABSTRACT

Cerebral microcirculation is critical for the preservation of brain health, and vascular impairment is associated with age-related neurodegenerative diseases. Because the retina is a component of the central nervous system, cellular changes that occur in the aging retina are likely relevant to the aging brain, and the retina provides the advantage that the entire vascular bed is visible, en face. In this study, we tested the hypothesis that normal, healthy aging alters the contractile vascular smooth muscle cell (VSMC) coverage of retinal arterioles. We found that aging results in a significant reduction of contractile VSMCs in focal patches along arterioles. Focal loss of contractile VSMCs occurs at a younger age in mice deficient in the senescence-associated protein, caveolin-1. Age-related contractile VSMC loss is not exacerbated by genetic depletion of insulin-like growth factor-1. The patchy loss of contractile VSMCs provides a cellular explanation for previous clinical studies showing focal microirregularities in retinal arteriolar responsiveness in healthy aged human subjects and is likely to contribute to age-related retinal vascular complications.

1. Introduction

Increasing evidence from clinical and experimental studies indicates that age-related microvascular dysfunction and microcirculatory damage play critical roles in a wide range of pathologies affecting the central nervous system (CNS) in older adults (Toth et al., 2017). It has become evident in recent years that aging causes similar functional and structural microvascular alterations throughout the CNS, which contribute to the pathogenesis of diseases ranging from vascular cognitive impairment to age-related macular degeneration. Because the retina is a tissue of the CNS, which projects out of the diencephalon and has similar embryonic origin as other parts of the brain, it shares a similar microcirculatory network with similar autoregulation, endothelial barrier function, as well as pathophysiology. Recent studies demonstrate that early retinal microvascular alterations can be used as biomarkers for different pathophysiologies affecting the brain, demonstrating hypertension and diabetes-induced microvascular lesions, predicting stroke risk, and microvascular consequences of Alzheimer's disease. In aging, retinal arterioles and brain vessels also exhibit similar functional and structural alterations, including impaired barrier function and blood flow regulation and reduced vascular branching (Catita et al., 2015; Chan-Ling et al., 2007; Feng et al., 2008; Grossniklaus et al., 2013; Hughes et al., 2006).

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Although aging-induced pathophysiological alterations of endothelial cells are well studied, the age-related changes in vascular smooth muscle cell (VSMC) phenotype are less understood. Previous studies demonstrate that aging impairs contractile function of resistance arterioles in the CNS, impairing spontaneous myogenic tone (Springo et al., 2015) and autoregulatory function of resistance arteries and their functional and structural adaptation to hypertension (Toth et al., 2013). Impaired VSMC contractile function of retinal arterioles has also been demonstrated in older adults (Gugleta et al., 2013; Kneser et al., 2009; Kotliar et al., 2008; Seshadri et al., 2016). Aging also promotes microvascular regression (Lynch et al., 1999) and compromises structural integrity of VSMC-containing arterioles, which contribute to increased prevalence of spontaneous rupture of these vessels (Toth et al., 2015b). Despite these advances, the underlying mechanisms by which aging affects arteriolar VSMCs are not well understood.

The present study was designed to test the hypothesis that aging alters the coverage of arterioles by contractile VSMCs in the CNS. To test our hypothesis, we analyzed α-smooth muscle actin (αSMA)-positive, contractile VSMC coverage in first-order arterioles in retinal whole mount preparations derived from young and aged C57BL/6 mice by immunohistochemistry. To determine whether aging promotes apoptosis in mural cells, activated caspase-3 was immunolocalized. To test the subhypothesis that circulating insulin-like growth factor-1 (IGF-1) deficiency exacerbates age-related changes in arteriolar contractile VSMC coverage, we used an established mouse model of isolated endocrine IGF-1 deficiency induced by adeno-associated viral knockdown of IGF-1 specifically in the mouse liver using Cre-lox technology (*Igf1*$^{f/f}$ + albumin promoter–driven Cre recombinase) (Ashpole et al., 2017; Tarantini et al., 2016a,b; Toth et al., 2014, 2015a). To test the corollary hypothesis that deficiency in the aging modulator caveolin-1 (Cav-1) exacerbates age-related changes in arteriolar contractile VSMC coverage, we used Cav-1 knockout (KO) mice and wild-type (WT) littermate controls.

2. Methods

2.1. Animals

All procedures were performed in accordance with the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the Institutional Animal Care and Use Committees of the University of Oklahoma Health Sciences Center. Experiments were performed on the following mouse strains: male C57Bl/6J mice; global Cav-1 KO (C57Bl6/129; stock #004585; The Jackson Laboratory) and littermate controls generated from heterozygous mating; αSMA–green fluorescent protein (GFP) reporter mice (C57Bl/6 background) (Kador et al., 2012); and global Cav-1 KO mice (C57BL/6J background; stock #007083) crossed with the αSMA-GFP reporter mice. To target IGF-1 production mice, homozygous for a floxed exon 4 of the *Igf1* gene (*Igf1*$^{f/f}$; in a C57BL/6 background) were crossed with mice expressing albumin-driven Cre recombinase, as previously described (Ashpole et al., 2016; Tarantini et al., 2016a). Knockdown of IGF-1 was verified by measuring circulating levels of IGF-1, as reported (Ashpole et al., 2016). Mice were housed in microisolation caging on a 12 hours light/12 hours dark cycle with free access to standard rodent chow (PicoLab Rodent Diet 20 5053, LabDiet, Land O'Lakes, Inc, St. Louis, MO, USA) and water.

2.2. Retinal whole mount preparation and immunohistochemistry

After euthanasia by CO_2 asphyxiation, eyes were enucleated and immersion-fixed in 4% paraformaldehyde and processed as previously described (Gu et al., 2014). Briefly, eyes were hemisected and retinas dissected under a stereomicroscope (Carl Zeiss Microscopy, Jena, Germany), permeabilized with 1% Triton X-100 in phosphate-buffered saline (PBS), blocked with 10% normal horse serum in 0.1% Triton X-100 in PBS, and incubated with the following primary antibodies at 4 °C overnight: hamster anti-CD31 (1:200; EMD Millipore, Billerica, MA or Developmental Studies Hybridoma Bank, University of Iowa), rabbit anti-Cav-1 (1:200; BD Pharmingen, San Diego, CA and 1:400; Cell Signaling Technology, Danvers, MA, USA), guinea pig anti-neuron/glial antigen-2 (NG2; 1:200; gift from Dr William Stallcup, Sanford Burnham Prebys Medical Discovery Institute), rabbit anti-type IV collagen (Millipore Sigma, Burlington, MA, USA), rabbit anti-cleaved caspase-3 (1:400; Cell Signaling Technology, Inc, Danvers, MA, USA), Cy3-conjugated anti-αSMA (clone 1A4; 1:250; Sigma-Aldrich). It has previously been established that cytoskeletal proteins are reliable markers to represent the VSMC contractile phenotype (Kocher et al., 1984; Owens, 1995), and we used αSMA as a contractile marker (Gabbiani et al., 1981).

After washing with 0.1% Triton X-100 in PBS (3 × 15 minutes), the retinas were incubated with the appropriate fluorophore-conjugated secondary antibodies (1:500; Life Technologies, Carlsbad, CA, USA) and/or 1:200 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA)] at 4 °C overnight. After another washing with 0.1% Triton X-100 in PBS, 4 to 5 radial cuts were made in the retinas, from the edge toward the optic nerve head, ganglion cell layer up, for flat-mounting in glycerol:PBS (1:1, v/v). Imaging was performed using the FV1200 confocal laser scanning microscope (Olympus, Tokyo, Japan), and images were processed with Photoshop CS5 (Adobe Systems, San Jose, CA, USA).

2.3. In vivo funduscopy

Young (4 months) and aged (19 months) αSMA-GFP mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, and pupils were dilated with 2.5% phenylephrine. Fluorescent fundus images were captured noninvasively using the Micron IV Retinal Imaging Microscope (Phoenix Research Labs, Pleasanton, CA, USA). After imaging, mice were euthanized and retinas collected for immunohistochemical analysis to compare in vivo GFP fluorescence with smooth muscle cell (SMC) coverage.

2.4. Morphometry

Coverage of retinal arterioles by contractile VSMCs was analyzed using ImageJ version 10.2 software (NIH, Bethesda, MD) in maximum projections of confocal image stacks of retinal arterioles that branch from the central retinal artery entering from the optic nerve head (analogous to "branch retinal arteries," labeled as "A" in Fig. 1A). CD31 or Collagen IV costaining was used to distinguish actual regions of contractile VSMC loss from artifactual tears/cuts in the vessels that occur during whole mount preparation. The areas of the gaps in αSMA coverage were assessed individually from each arteriole, and their sum was subtracted from the total coverage per vessel to derive the αSMA coverage/vessel area ratio. The number of gaps in coverage were also quantified and normalized to arteriolar area. We also quantified immunoreactivity of cleaved caspase-3, αSMA, and CD31 in retinal arterioles from young and old mice and also analyzed the intensity of cleaved caspase-3 immunoreactivity in αSMA-covered and uncovered regions using ImageJ.

2.5. Statistical analyses

The total area of each arteriole (3–7/retina) was individually assessed using ImageJ software. The area of αSMA coverage on the same arterioles was then assessed by subtracting the combined areas of the gaps in immunostaining from the total area. The αSMA