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Angiography reveals novel features of the retinal vasculature in healthy and diabetic mice

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ABSTRACT

The mouse retina is a commonly used animal model for the study of pathogenesis and treatment of blinding retinal vascular diseases such as diabetic retinopathy. In this study, we aimed to characterize normal and pathological variations in vascular anatomy in the mouse retina using fluorescein angiography visualized with scanning laser ophthalmoscopy and optical coherence tomography (SLO-OCT). We examined eyes from C57BL/6J wild type mice as well as the *Ins2*^{Akita} and Akimba mouse models of diabetic retinopathy using the Heidelberg Retinal Angiography (HRA) and OCT system. Angiography was performed on three focal planes to examine distinct vascular layers. For comparison with angiographic data, *ex vivo* analyses, including Indian ink angiography, histology and 3D confocal scanning laser microscopy were performed in parallel. All layers of the mouse retinal vasculature could be readily visualized during fluorescein angiography by SLO-OCT. Blood vessel density was increased in the deep vascular plexus (DVP) compared with the superficial vascular plexus (SVP). Arteriolar and venular typologies were established and structural differences were observed between venular types. Unexpectedly, the hyaloid artery was found to persist in 15% of C57BL/6 mice, forming anastomoses with peripheral retinal capillaries. Fluorescein leakage was easily detected in Akimba retinae by angiography, but was not observed in *Ins2*^{Akita} mice. Blood vessel density was increased in the DVP of 6 month old *Ins2*^{Akita} mice, while the SVP displayed reduced branching in precapillary arterioles. In summary, we present the first comprehensive characterization of the mouse retinal vasculature by SLO-OCT fluorescein angiography. Using this clinical imaging technique, we report previously unrecognized variations in C57BL/6J vascular anatomy and novel features of vascular retinopathy in the *Ins2*^{Akita} mouse model of diabetes.

1. Introduction

Alterations in the retinal vasculature are a common feature in ocular pathology arising from a diverse number of causes, including diabetic retinopathy (DR), retinopathy of prematurity, retinal vein occlusion, macular telangiectasia and sickle cell disease (Cheung et al., 2010; Robinson and Halpern, 1992; Sarks et al., 1997; Serjeant et al., 1986; Smith, 2003). Small animal models of retinal

Abbreviations: DR, diabetic retinopathy; DVP, deep vascular plexus; EGFP, enhanced green fluorescent protein; HRA, Heidelberg Spectralis Retinal Angiography; IVP, intermediate vascular plexus; PBS, phosphate buffered saline; PDGF-Rβ, platelet derived growth factor-receptor-β; SLO-OCT, scanning laser ophthalmoscopy and optical coherence tomography; SMA, smooth muscle actin; SVP, superficial vascular plexus; VEGF, vascular endothelial growth factor; WT, wild type.

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vascular disease are commonly used for the study of pathophysiology and development of treatment strategies for these blinding diseases. A number of transgenic and knockout mice, as well as toxin-induced models of disease, such as diabetes, have been reported to display retinal vascular pathology (Ramos et al., 2013). While mice often exhibit early features of vascular distress, including changes in vascular permeability, leukostasis and capillary drop out (Cheung et al., 2005; Hammes et al., 2002; Joussen et al., 2004), these models rarely display late features of retinopathy as seen in human patients clinically, such as neovascularization and cystoid oedema (McLenachan et al., 2013; Ramos et al., 2013). Therefore, the discovery of mouse models that exhibit vascular features resembling human disease remains a high priority in retinopathy research.

A number of advanced imaging technologies have been used to study the rodent retinal vasculature, including confocal scanning laser microscopy (Mendes-Jorge et al., 2012; Paques et al., 2006; Ramos et al., 2013; Ritter et al., 2005) as well as scanning laser ophthalmoscopy and optical coherence tomography (SLO-OCT) (Ali Rahman et al., 2011; McLenachan et al., 2013; Paques et al., 2007, 2006; Rakoczy et al., 2010; Zhi et al., 2014). Since SLO-OCT is commonly used in screening, diagnosis and staging of retinopathy in human, it is important that this technique is also used for the characterisation of mouse models of retinal vascular disease. The anatomy of retinal blood vessels in the mouse has been previously described using confocal scanning laser microscopy (Paques et al., 2006; Ramos et al., 2013) and optical microangiography (Zhi et al., 2014), however a comprehensive description using SLO-OCT is currently unavailable. Therefore, we aimed to determine if the various layers of the mouse retinal vasculature can be visualized through fluorescein angiography using a commercially available clinical SLO-OCT system, the Heidelberg Spectralis Retinal Angiography (HRA) device and to identify the normal and pathological variations present in the retinal vasculature of healthy and diabetic mice.

We examined retinal vascular anatomy in a large cohort of C57BL/6J mice using the HRA. We demonstrate the visualization of all layers of the mouse retinal vasculature by HRA and determine the normal variations in vessel number and branching patterns. Several typologies of arterioles and venules are identified and molecular differences between them demonstrated using specific markers of the vessel wall. Analysis of vascular densities revealed large differences in vessel density between different layers of retinal vasculature, highlighting a potential source of experimental error in previous angiographic studies. Unexpectedly, we detected the persistence of the hyaloid artery in 15% of wild type C57BL/6 mice and these vessels formed anastomoses with peripheral retinal vasculature, revealing a previously unrecognized anatomical variation in these mice. Fluorescein leakage could be easily detected by HRA in the Akimba mouse model of VEGF-induced retinal neovascularization, however, we did not observe signs of leakage in diabetic *Ins2*^{Akita} mice. Instead, other signs of vascular pathology were observed in *Ins2*^{Akita} mice at 6–12 months of age, including beading of major vessels, neovascularization in the capillary bed and pruning of precapillary arteriole branches. Together, our results provide new insights into the acquisition and interpretation of fluorescein angiography data using an SLO-OCT system, the heterogeneity of the normal mouse retinal vasculature and the pathophysiology of diabetic retinopathy in the *Ins2*^{Akita} mouse.

2. Material and methods

2.1. Animals

In the present study we used normal wild type (WT) C57BL/6J mice, *Ins2*^{Akita} mice (Yoshioka et al., 1997), Akimba mice (Rakoczy et al., 2010) and β-actin/EGFP mice (Okabe et al., 1997) aged from 9 to 25 weeks old. A small group of aged (12–13 month old, n = 3) WT and *Ins2*^{Akita} mice was also examined. Animals were bred at the Animal Resources Centre (Murdoch University, WA) and the Animal Facility of the Universitat Autònoma de Barcelona (UAB, Barcelona, Spain). Mice were maintained on a 12-h day/night cycle, with free access to food and water. Principles of laboratory animal care (NIH publication no. 85-23) were followed. All procedures were performed in accordance with the Declaration of Helsinki and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by The University of Western Australia Animal Ethics Committee and the Ethics Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona.

2.2. Scanning laser ophthalmoscopy and optical coherence tomography

Clinical imaging was performed as previously described (McLenachan et al., 2013). Mice were anaesthetized using ketamine/xylazine and retinal imaging was performed using the Spectralis Heidelberg Retinal Angiography and Optical Coherence Tomography (HRA-OCT) imaging system (Heidelberg Engineering, Heidelberg, Germany) with a 25-diopter lens fitted on a 30° field of view objective lens. For angiography, Spectralis HRA-OCT was operated in fluorescence mode and images collected using the high resolution mode. For OCT, volume scans were acquired from the posterior pole as well as from peripheral areas. Eyes were moistened with eye drops containing tropicamide and lignocaine to dilate the pupils. A custom contact lens was placed on the corneas to prevent drying and the resultant temporary cataract developing during imaging. After fundus HRA and OCT images had been acquired, 50 μl of 10% sodium fluorescein was injected subcutaneously and fluorescein angiography was performed using the fluorescence mode of the HRA system. After retinal imaging, mice were allowed to recover and kept for a further 4 days. Animals were sacrificed at 9, 14, 17, 21 and 25 weeks of age.

2.3. Immunohistochemistry

Eyes embedded in paraffin were sectioned (3 μm) along the eye axis through the optic disc and cornea, deparaffinised and rehydrated. For whole-mount preparations of retinae, eyes were fixed in 10% neutral buffered formalin for 2 h at 4 °C. The retinae were dissected as previously described (McMenamin, 2000). After they were washed in PBS, paraffin sections and free floating whole-mount retinas were incubated overnight with the following markers: anti-collagen IV (Millipore, Darmstadt, Germany) at 1:20 dilution; anti-platelet derived growth factor-receptor-β (PDGF-Rβ) (Abcam, Cambridge, UK) at 1:100 dilution; anti-NG2 (Millipore) at 1:200 dilution; anti-α-smooth muscle actin (α-SMA) (Abcam) at 1:100 dilution and rhodamine-labelled phalloidin, a cyclic peptide isolated from *Amanita phalloides* mushroom that has a high-affinity for F-actin (Sigma–Aldrich, St. Louis, Missouri, USA) at a concentration of 5 μg/ml. Retinas were washed several times in PBS and then incubated overnight at 4 °C with specific secondary antibodies: anti-goat IgG-biotinylated (Sta. Cruz Biotechnology, Inc., Sta. Cruz, California, USA) and anti-rabbit Alexa Fluor 568 (Invitrogen, Carlsbad, USA) both antibodies at 1:100 dilution. Once retinas were washed in PBS, Streptavidin Alexa Fluor 488 was used as fluorochrome (Invitrogen) diluted at 1:100. β-actin/EGFP mouse retinae were dissected, fixed and mounted for the direct visualization of EGFP fluorescence. Microscopic analysis was performed with the laser scanning confocal microscope (TCS SP2; Leica