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Alterations to vascular endothelium in the optic nerve head in patients with vascular comorbidities

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ABSTRACT

Vascular comorbidities are inherently linked to the pathogenesis of central retinal artery occlusion (CRAO) and central retinal vein occlusion (CRVO). However, the endothelial-mediated pathogenic mechanisms that precede, and therefore modulate, luminal occlusion have not been clarified. The aim of this study was to delineate the pattern of endothelial morphometric alteration in the central retinal artery and vein in patients with vascular comorbidities. Eyes with a previous history of vascular occlusion were not included in this study in order to avoid the confounding effects of post-occlusion endothelial changes. This study also sought to determine if vascular comorbidities had a disparate effect on arterial and venous endothelium in the optic nerve head. Comparisons were made between 13 human eyes from patients with vascular comorbidities and 22 control eyes from patients with no known systemic disease. Novel micro-cannulation techniques developed in our laboratory were used to label the cytoskeleton and nuclei of endothelial cells in the central retinal artery and vein following which images were captured using confocal microscopy. Endothelial and nuclear morphometric parameters were quantified in different laminar regions of the optic nerve head. F-actin stress fibre expression was also quantified. Analysis of covariance was used to determine statistical differences between the two groups. Interestingly, age did not influence endothelial morphometry, nuclear morphometry or f-actin expression in central retinal vessels. There were also no arterial endothelial differences between control and disease groups in any laminar region. Endothelial f-actin stress fibre expression increased significantly in the central retinal vein in patients with vascular comorbidities. The greatest change in these eyes was found to occur at the posterior lamina cribrosa. Increased venous endothelial f-actin stress fibre expression may reflect vascular comorbid disease-induced alterations to hemodynamic properties and coagulation cascades in the central retinal vein. The posterior lamina may be an important site for thrombus formation in CRVO as venous endothelia in this region are most influenced by the presence of vascular comorbidities. The findings of this study suggest that the role of endothelial dysfunction in CRVO and CRAO pathogenesis could be different.

1. Introduction

Vascular comorbidities are inherently linked to the development and progression of central nervous system disease (Gorelick et al., 2011; Marrie et al., 2010). Various epidemiological studies have shown that the prevalence of central retinal artery occlusion (CRAO) and central retinal vein occlusion (CRVO) are greater in patients with vascular comorbidities (Cheung et al., 2008; Rudkin et al., 2010; Wong et al., 2005). However, the endothelia-mediated pathogenic mechanisms that underlie each of these diseases remain unclarified. Due to the unique hemodynamic properties of the central retinal artery and vein there is considerable

Abbreviations: CRAO, central retinal artery occlusion; CRVO, central retinal vein occlusion; *e*, distance from cell apex to nucleus in the downstream direction of blood flow; ANCOVA, analysis of covariance.

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debate concerning the cellular influence of vascular comorbidities upon each of these circulations. Specifically, it is unknown if systemic vascular diseases have a similar, or disparate, effect on arterial and venous endothelia in central retinal vessels. A detailed, cellular-level examination of central retinal vessels in patients with vascular comorbidities may help define some of the pathogenic mechanisms involved in CRAO and CRVO. Such knowledge may also provide insights into cellular pathways that could be targeted therapeutically for the management of retinal vascular disease.

Vascular endothelial cells play a critical role in ocular health and disease (Aird, 2007, 2008). Endothelial cells modulate regional homeostasis by controlling blood flow and modifying the behaviour of signalling cascades that regulate thrombosis and inflammation (Pearson, 1994; Pober and Sessa, 2007). Pathological states that perturb vascular hemodynamic properties induce changes to endothelial morphology as well as alterations to the expression of intracellular cytoskeleton proteins (Dewey et al., 1981; McCue et al., 2004; Nerem et al., 1981). These parameters can be reliably quantified and subsequently used to infer knowledge about disease-induced vascular alterations that cannot otherwise be measured *in vivo* (DeMaio et al., 2004; Kang et al., 2011; Sumagin et al., 2008). Our previous study demonstrated significant endothelial morphometric variations across the normal human optic nerve head and identified important correlations between endothelial morphometry and predicted tissue pressures in each laminar region (Balaratnasingam et al., 2009b; Kang et al., 2011). Other experimental studies, involving non-ocular circulations, have shown that endothelial modifications are specific to the form of vascular insult (Bonetti et al., 2003; Butler et al., 2001; Califano and Reinhart-King, 2010; Chiu and Chien, 2011; Hsiai et al., 2002). Examination of optic nerve head endothelium in patients with vascular comorbidities may therefore provide valuable insights into endothelia-mediated mechanisms that underlie retinal vascular occlusion. It may also help identify important patho-physiological distinctions between CRAO and CRVO.

This report utilises our novel perfusion methodology to document the morphometric characteristics of artery and venous endothelium in the human optic nerve head in patients with and without vascular comorbidities. The effect of vascular comorbid disease on endothelial f-actin stress fibre expression is also quantified. We hypothesise that vascular comorbidities will induce an alteration in endothelial morphometry and stress fibre expression in the central retinal artery and vein. The aim of this study is to identify endothelia-mediated disease mechanisms that precede, and therefore may be important, in the pathogenesis of CRAO or CRVO.

2. Materials and methods

This study was approved by the human research ethics committee at The University of Western Australia. All human tissue was handled according to the tenets of the Declaration of Helsinki.

2.1. Human donor eyes

All eyes in this study were from donors with no history of ocular disease. 13 eyes from 9 donors with vascular comorbid disease and 22 eyes from 14 donors with no known vascular disease were used for this study. Eyes in the latter group were used in our previous publication (Kang et al., 2011) with the addition of two eyes from a 28 year-old donor who died in a motor vehicle accident. In this report, the vascular comorbid disease group is referred to as the 'disease' group and eyes from normal donors are referred to as the 'control' group. The co-morbidities of individual donors in the disease group and their cause of death are provided in Table 1. All eyes were obtained from the Lions Eye bank of Western Australia (Lions Eye Institute, Western Australia) or Donate Life Australia.

2.2. Perfusion technique for optic nerve endothelium labelling

Perfusion-based immunohistochemical techniques, developed in our laboratory, were used to achieve targeted endothelial labelling of the central retinal vasculature (Yu et al., 2010a, 2010b, 2012). In brief, a glass pipette was used to cannulate the central retinal artery of enucleated eyes. Residual blood in the retinal circulation was then washed out with oxygenated Ringer's solution and 1% bovine serum albumin. After the Ringer's wash, 4% paraformaldehyde in 0.1 M phosphate buffer and 0.1% Triton-X-100 in 0.1 M phosphate-buffered solution were sequentially perfused to achieve fixation and permeabilisation, respectively, of endothelial cell membranes. Actin microfilament and endothelial nuclei were subsequently labelled by perfusion with a mixture of phalloidin conjugated to Alexa Fluor 546 (30 U; A22283; Invitrogen, Carlsbad, CA) and nucleus probe (bisbenzimide H33258; 1.2 μg/mL; Sigma–Aldrich, St. Louis, MO). The flow rate of perfusate was controlled with syringe pumps and the perfusion pressure was monitored during all stages of the perfusion. The eye was then immersion fixed in 4% paraformaldehyde before sectioning.

2.3. Tissue preparation

Tissue for cryosectioning was mounted in optimal cutting temperature compound (OCT; Tissue-Tek 4583, Product No. 62550-12; Sakura, Tokyo, Japan) and longitudinally sectioned on a cryotome set at −30 °C. Sections were 12 μm in thickness. The

Table 1
Demographic details of the disease group.

Patient ID	Sex	Age	Eye	Vascular comorbidities	Cause of death	Death-to-enucleation time (hrs)
A	M	49	R	Diabetes	Myocardial infarction	9.5
B	M	51	R + L	Hypertension Atherosclerosis	Myocardial infarction	9.5
C	M	61	R + L	Hypertension Smoking	Myocardial infarction	10
D	M	75	L	Hypertension Smoking	Subarachnoid haemorrhage	5
E	M	58	R + L	Atherosclerosis Smoking	Subarachnoid haemorrhage	9
F	M	73	L	Hypertension Atherosclerosis Dyslipidemia	Abdominal aortic aneurysm rupture	15
G	M	51	L	Diabetes Atherosclerosis	Myocardial infarction	19
H	M	53	R + L	Atherosclerosis	Myocardial infarction	19
I	M	74	L	Diabetes	Congestive heart failure	10

Vascular comorbidities and cause of death for each optic nerve donor are provided. All donors with atherosclerosis had clinical manifestations in the form of coronary artery disease or carotid artery stenosis, requiring surgical intervention. Sex (M = male or F = female); age (years); eye side (R = right or L = left).