SMA+ perivascular cells evaluation in VEGF induced blood-retinal barrier breakdown in rabbit model.

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Background

Diabetic retinopathy (DR) is a progressive disease that develops from asymptomatic vascular and neuronal changes into vision threatening diabetic macular edema (DME) and proliferative DR (PDR)[1]. DME is caused by loss of the blood-retinal barrier (BRB), but the detailed molecular mechanisms underlying increased permeability of the BRB in DR remain not fully understood. The BRB is composed of endothelial cells, pericytes and glial cells that form the neurovascular unit[2]. The presence and crosstalk between all cell types in this unit is crucial for barrier maintenance. It has been shown that VEGF coordinates interactions between endothelial and perivascular cells. In animal models, sustained doses of VEGF injected into the eyes results in vessel dilatation, tortuosity and vascular retinal permeability[3] with pericyte loss[4]. Technically, this model is not a specific DR model however it mimics the early stage of DR and can be used for potential treatment to protect pericytes from dysfunction or loss. The aim of the present study was to evaluate perivascular cell pattern in rabbit induced blood-retinal barrier breakdown model. We used two approaches to characterize the model: functional approach (retinal permeability) and morphological approach (vessel integrity).

Materials and methods

Animals

Ten adult male pigmented rabbits were used in the study. Animals were handled and cared for according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction

Increase in retinal vascular permeability was induced by a single 50 µL intravitreal injection of 500 ng rhVEGF165 (diluted in PBS with carrier protein) into the mid-vitreous of the right eyes using a Myjector syringe (29-G needle) on anesthetized animals.

Fluorescein angiography and scanning ocular fluorometry

Forty-eight hours after induction, rabbits were anesthetized and 50 mg/kg sodium fluorescein was injected via the marginal vein. Pupils were dilated and late phase angiograms were obtained 5 min later. Ocular fluorescein levels within the eye were measured using the Fluorotron Master (Ocumetrics) sixty minutes following fluorescein injection.

Immunohistochemistry of flat-mounted retina

The eyes were fixed in 4% buffered formaldehyde for 4h at room temperature. The flat-mounted retina were then rinsed with PBS and a double staining with biotinylated Isolectin B4 (Sigma; ref L2140, 1:50) and anti-SMA antibody coupled FITC (Sigma; ref: F3777, 1:50) was done.

An evaluation of the pericytes on flat-mounted retinas was performed by separating vessels into 3 area from the optic disc to the periphery (figure 3).

The SMA+ perivascular cell coverage was estimated by calculating the ratio between the total surface of the vessels and the surface of SMA positive labelling.

Results

A-Functional approach

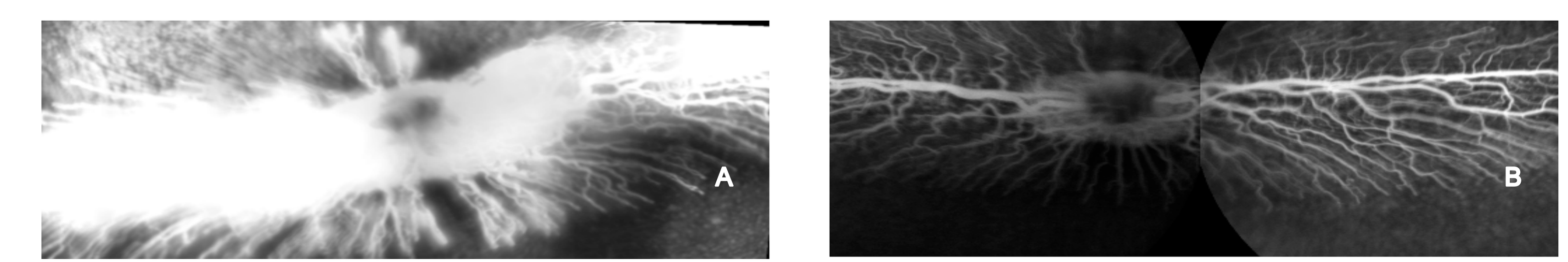

Figure 1. Representative fluorescein angiography images from VEGF-induced eye (A) and control eye (B).

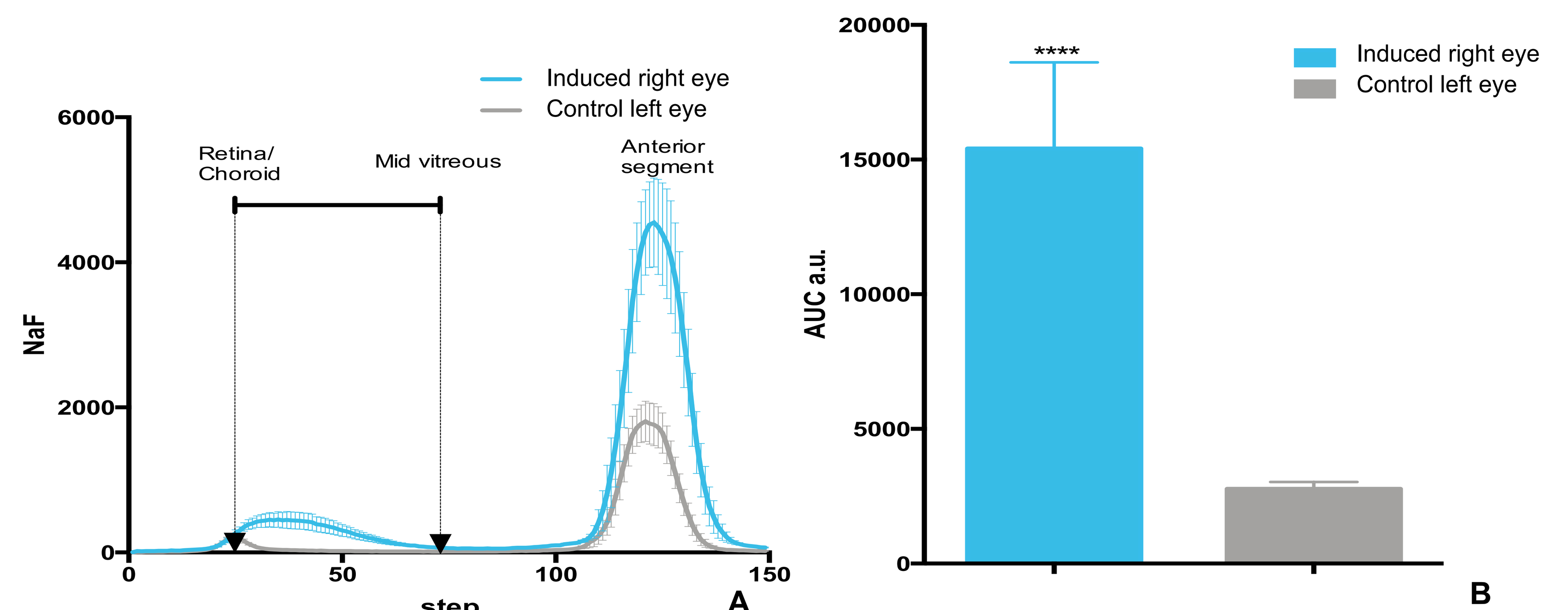

*Figure 2. Representative fluorometry scans (A) and quantification of fluorescein level in retina-vitreous compartment by integration of area under the curve (B). Bars represent mean ± SEM (n=10/group). Data were compared with non parametric Mann and Whitney test (**** = p<0.0001).*

Fluorescein angiography (figure 1)

Fluorescein angiography performed 48h after induction showed massive leakage of fluorescein from retinal vessels in VEGF-induced eyes compared to control eye. No individual vessels could be observed in the induced eye shortly after the fluorescein injection (figure 1A).

Scanning ocular Fluorometry (figure 2)

Fluorometric data are presented in units of fluorescence and represent a series of measurements (148 steps, 250 µm/step) along the optical axis from cornea to retina (figure 2A). Data were plotted and the area under the curve (AUC) was measured from vitreoretinal compartment. AUC is shown in arbitrary units of fluorescein x step. Relative to control left eyes, intravitreal injection of VEGF cause a 5 fold increase in vitreoretinal compartment (figure 2B).

B-Morphological approach

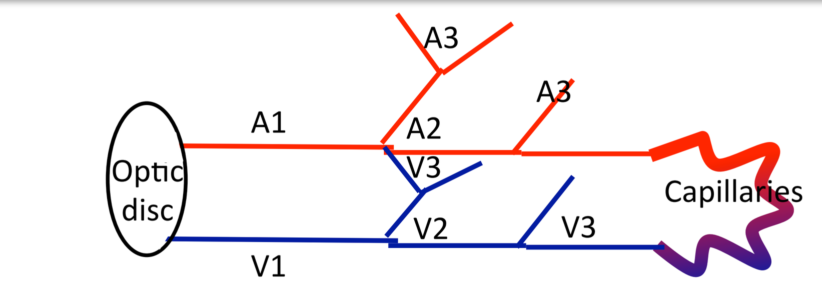

Figure 3. Schematic diagram of the microvascular tree from the optic disc to the periphery. A: arteriol, V: venule. Area are based on the branching pattern

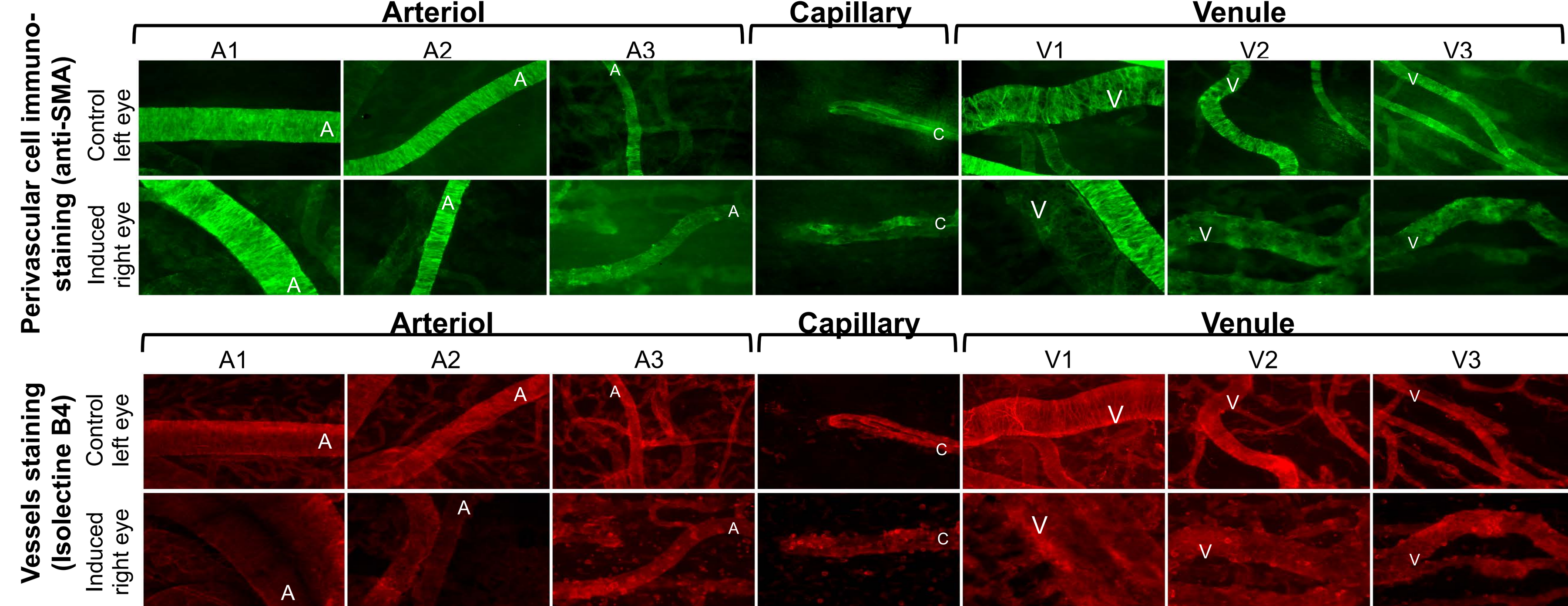

Figure 4. Representative anti-SMA (green) staining and Isolectin B4 (red) staining of retinal vasculature (x200 magnification). A: arteriol, C: capillary, V: venule

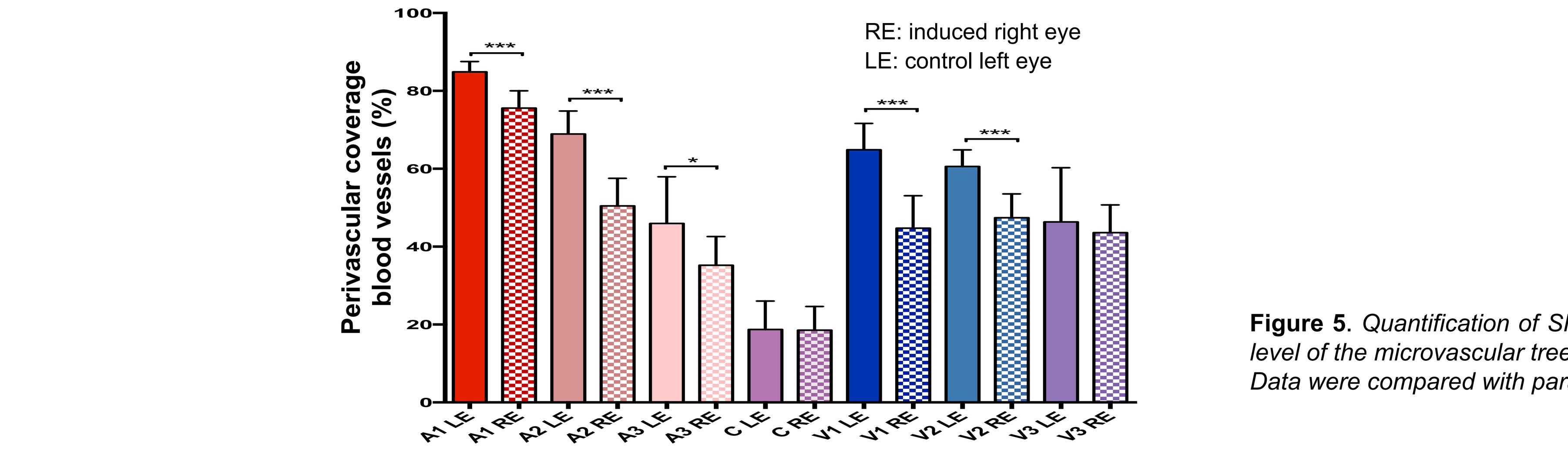

*Figure 5. Quantification of SMA+ cells coverage of vessels at the different level of the microvascular tree. Bars represent mean ± SEM (n=10/group). Data were compared with parametric t-test (*** = p<0.001)*

Immunohistochemistry of flat-mounted retina

In the control left eye, at the level of primary (A1, V1) or secondary (A2, V2) branching, the SMA+ perivascular cells have a typical morphological appearance that encircle the vessels giving rise to striated appearance (figure 4). The SMA+ perivascular cells are tightly packed at high density. At the level of tertiary branching, The SMA+ perivascular cells are distributed along the vessels at a lower density with more space between cells. More distally, at the level of capillary, few SMA+ cells were observed.

Intravitreal injection of VEGF result in significant reduction of SMA+ perivascular cell coverage in retinal vasculature at the level of primary and secondary branching (figure 5). Approximately 10 to 20% of SMA+ cells were lost.

Conclusion

This study shows that VEGF induced a retinal permeability and indicates that VEGF decreased SMA+ cells coverage of both arteriolar and venular tree in rabbit suggesting that this model can be used as a DR model.

1. Hammes et al., 2011. Diabetes. 2011 Jan;60(1):9-16. **2.** Klaassen et al., 2013. Prog Retin Eye Res. 2013 May;34:19-48. **3.** Edelman et al. Exp Eye Res. 2005 Feb;80(2):249-58. **4.** Cao et al. Proc Natl Acad Sci U S A. 2010 Jan 12;107(2):856-61

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