Blood-ocular barriers in health and disease. Light and electron microscopic studies of the eye

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CHAPTER IX
NEW INSIGHTS INTO THE PATHOGENESIS OF CAPILLARY NON-PERFUSION IN THE RETINA: ENDOTHELIAL CELL HYPERTROPHY INDUCED BY VASCULAR ENDOTHELIAL GROWTH FACTOR

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ABSTRACT

Objective: To investigate the mechanism leading to capillary non-perfusion of the retina, in a monkey model of vascular endothelial growth factor (VEGF)-induced retinopathy in which capillary closure occurs in a late stage after VEGF treatment.

Methods: Two monkeys received 4 intravitreous injections of 0.5 µg VEGF in one eye and of PBS in the other eye, and were sacrificed at day 9. After perfusion and enucleation, retinal samples were snap frozen for immunohistochemistry with the pan-endothelial cell marker CD31 or fixed for morphometric analysis at the light and electron microscopic level.

Results: At the light microscopic level, all capillaries in the retina of VEGF-injected eyes displayed hypertrophic walls with narrow lumina. In a quantitative analysis of the deep capillary plexus in the inner nuclear layer, VEGF-injected eyes had a significant 5 to 7-fold decrease in total capillary luminal volume. CD31 staining showed that this decrease was not accompanied by a change in the number of capillaries. Electron microscopy revealed that the luminal volume of individual capillaries of the inner nuclear layer of VEGF-injected eyes was significantly decreased due to a 2-fold hypertrophy of the endothelial cells.

Conclusions: Luminal narrowing caused by endothelial cell hypertrophy occurs in the deep retinal capillary plexus in VEGF-induced retinopathy in monkeys. This suggests a causal role of endothelial cell hypertrophy in the pathogenesis of VEGF-induced retinal capillary closure. A similar mechanism may operate in retinal conditions in humans associated with ischemia and VEGF over-expression.

Clinical relevance: Capillary non-perfusion occurs in diabetic retinopathy (DR) and other ischemic diseases associated with over-
expression of VEGF. VEGF-induced endothelial cell hypertrophy may be causative for capillary closure in these diseases.

INTRODUCTION
Capillary non-perfusion is a hallmark of diabetic retinopathy (DR), retinal vein occlusions, and other retinal ischemic diseases. In the pathogenesis of DR, capillary closure of the inner retinal capillary plexus is an early critical event, preceding macular edema and neovascularization. What leads to the occlusion and non-perfusion of retinal capillaries in the early stages of DR (background DR) remains unknown. Some authors have attributed a role to increased venous blood flow and impaired autoregulation in the diabetic retinal vasculature and have suggested that the resulting increase in shear stress in the microvasculature, combined with endothelial dysfunction and platelet aggregation, is responsible. Others have allocated a role to endothelial and leukocyte activation resulting in leukocyte adhesion, leukostasis and subsequent capillary closure. Retinal ischemia caused by capillary non-perfusion is associated with the production of vascular endothelial growth factor-A (VEGF). This permeability and angiogenic factor is believed to be responsible for retinal microvascular leakage and neovascularization in DR. In a model of VEGF-induced retinopathy in monkey eyes, capillary non-perfusion was observed in a late stage, after repeated intravitreous VEGF injections. This suggests that VEGF itself may cause this phenomenon, possibly leading to a vicious circle in human ischemic retinal disease. In a similar model in the rat eye, VEGF leads to upregulation of intercellular adhesion molecule-1 (ICAM-1) and leukostasis, supporting a possible role of leukocyte adhesion as a mechanism of VEGF induced capillary non-perfusion.

As the possibility exists that increased ICAM-1 expression and leukostasis in the retinal vessels are manifestations of capillary obstruction rather than its primary initiator, we investigated in the present study the early effects of VEGF on retinal capillaries in a monkey model of VEGF-induced retinopathy.

MATERIAL AND METHODS

VEGF-induced retinopathy in monkeys
Two Cynomolgus monkeys (Macaca fascicularis), a 15-year old male and a 5-year old female, were used for these experiments. Both animals had been used for behavioral studies in the past. All experiments were carried out in accordance with the resolution on the use of animals for research of the Association for Research in Vision and Ophthalmology as well as in accordance with the guidelines established for animal care at the University of Nijmegen, The Netherlands.
The animals received four injections with PBS (50 μl) through the pars plana into the center of the vitreous using a 30-gauge needle in the left eye (days 0, 2, 4, 7), and four injections with bioactive human recombinant VEGF-A (Harbor Bio-products, Norwood, USA, 0.5 μg in 50 μl PBS) in the right eye (days 0, 2, 4, 7). Before intravitreal injection, 20 mg/kg ketamine hydrochloride, 0.005 mg/kg acepromazine and 0.03 mg/kg atropine sulfate was given intramuscularly for general anesthesia. The animals were killed on day 9 with an intravenous overdose of pentobarbital. Subsequently, the head region of the animals was perfused through the abdominal aorta with PBS (37°C, pH 7.4) for 10 minutes at a controlled perfusion pressure of 70-80 mmHg and the eyes were enucleated. The eyes were dissected and samples of retina were either snap frozen in liquid
nitrogen for immunohistochemistry or fixed at room temperature for 1 hour by immersion in a mixture of 1.25% glutaraldehyde and 1% paraformaldehyde in PBS (pH 7.4) for light and electron microscopy.

**Light microscopic evaluation of retinal capillaries**

*Immunohistochemistry*
To determine qualitative changes induced by VEGF in the retinal vasculature, immunohistochemistry for the pan-endothelial cell marker CD31 (EN4, Monosan, Uden, The Netherlands, 1/100) was employed. Cryosections of retina were fixed in cold acetone for 10 minutes, post-fixed for 2 minutes in Zamboni...
Fig. 3: Diagram of the total luminal area (μm² ± standard error of mean) of capillaries in the inner nuclear layer, per millimeter retina, in the PBS-injected eyes and in the VEGF-injected eyes of both monkeys. n = number of retinal sections examined.

fixative (2% paraformaldehyde in a saturated picric acid solution), and stained by an indirect immunoperoxidase procedure. All incubations, except the overnight incubation, were performed at room temperature. Sections were pre-incubated with 10% normal goat serum in PBS for 15 minutes (Jacksons Immuno Research Laboratories, West Grove, Pennsylvania, USA) in order to reduce non-specific staining and then incubated overnight at 4°C with monoclonal antibody EN4 (Monosan, Uden, The Netherlands, 1:1000). Sections were washed, incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (GAM-PO, DAKO, Glostrup, Denmark, 1:100) for 30 minutes. Sections were washed and staining was performed using 3-3-di-amino benzidine (10 mg/ml, supplemented with 0.01% H₂O₂). The reaction was terminated by rinsing the sections with water. Counterstaining was performed with haematoxylin. Sections were washed and coverslipped in glycerol/glycerine (Sigma, St Louis, Missouri, USA). In control sections the first antibody was omitted.

Morphometric analysis of retinal capillary lumina
To study the effect of VEGF on the retinal vasculature, we determined the total vascular luminal area in retinal cross sections by a morphometric approach. We chose to only analyze the capillary plexus of the inner nuclear layer, as these capillaries are numerous and of homogeneous size and, by their anatomic localization, could be measured reproducibly in all sections. After fixation with 1.25% glutaraldehyde and 2% paraformaldehyde in PBS (pH 7.4), samples of retina were washed with PBS, postfixed for 45 minutes in phosphate buffered 1% osmiumtetroxide (OsO₄), dehydrated in a series of graded ethanols (3x5 minutes each) and finally embedded in epoxy resin. To calculate the capillary luminal area per millimeter retina the following procedure was followed. Six
Fig. 4: Electron micrographs of capillaries in the inner nuclear layer of the retina in the PBS-injected monkey eye (Fig 4A, 4B), and VEGF-injected monkey eye (Fig 4C, 4D). Note the small lumen (arrows) and endothelial cell (e) hypertrophy in the retinal capillaries of the VEGF-injected eye. I = lumen.

Semithin (1 μm) sections (>0.2 mm distant from each other) of two different retina samples of the posterior segment of each eye were cut, stained with toluidin blue, and examined and photographed using a light microscope. In these semithin sections, the area occupied by the lumen of all visible capillaries in the inner nuclear layer and the length of each retina section were determined. These measurements were performed on a VIDAS image analysis system (Kontron, Munich, Germany).
**Electron microscopic evaluation of inner nuclear layer capillaries**

In this part of the study, the endothelial cell area and luminal area of individual cross sections of capillaries in the inner nuclear layer were assessed. We chose again to only analyze the capillary plexus of the inner nuclear layer, as these capillaries, by their anatomic localization, could be measured reproducibly in all sections. Ultrathin sections of epoxy resin embedded retinal samples were stained with uranyl acetate and lead citrate and examined and photographed in a Philips EM201 electron microscope (Philips, Eindhoven, The Netherlands). Of each eye, all visible cross sections of capillaries in the inner nuclear layer were photographed (n >10). To calculate the area of the endothelial cells and lumen of these capillaries, a square grid of lines 2.5 mm apart was used. This grid was superimposed over each capillary in the electron micrographs and points overlying the lumen and the endothelial cells of the capillaries were counted according the point-counting method of Weibel. From these measurements, the relative volumes occupied by the lumen and endothelial cells were calculated for each individual capillary. Furthermore, the number of lateral inter-endothelial cell borders was determined in each individual capillary to estimate the number of endothelial cells.

**Statistical analysis**

The means (±standard error of mean (SEM)) of the calculated total area of capillary lumen per millimeter of retina (of six semithin sections for each eye) were compared between VEGF- and PBS-injected eyes of each monkey. From the electron microscopy observations on individual capillaries, for each eye, the means of volumes occupied by the lumen, endothelial cells, and the mean number of endothelial cell borders per capillary were calculated. The Mann-Whitney U-test was used to determine the level of significance for all measurements between PBS- and VEGF-injected eyes of each monkey. The level of significance was set at 5%.

**RESULTS**

**Light microscopic evaluation of retinal capillaries**

Immunohistochemical staining for the pan-endothelial cell marker CD31 demonstrated that there was no qualitative difference between VEGF-injected eyes and PBS-injected eyes in vascularization in the inner nuclear layer and other parts of the retina. However, in the VEGF-injected eyes most capillaries in the inner retina, both in the primary and in the deep vascular network, seemed to be enlarged as compared to capillaries in the PBS-injected eyes (exemplified in Figure 1).

In semithin sections of retinas of VEGF-injected eyes a marked decrease in the size of vascular lumina of retinal capillaries in all layers of the inner retina was
observed when compared to the PBS-injected eyes (exemplified in Figure 2). This was further analyzed by morphometric measurements of the deep capillary plexus of the inner nuclear layer. This retinal layer was chosen as its contains a relatively homogeneous population of capillaries which can be easily identified at the light and electron microscopic level. This morphometric analysis showed that in the VEGF-injected eyes, the total luminal area of capillaries in the inner nuclear layer (per mm retina) was significantly decreased by 5 to 7-fold (P < 0.01), when compared to the capillary luminal area per millimeter retina in the PBS-injected eyes (Figure 3).

No leukocytes or other blood cells were observed in the retinal capillaries of either VEGF- or PBS-injected eyes.

**Electron microscopic analysis of retinal capillaries**

Electron microscopic analysis was limited to the true capillaries of the inner nuclear layer. In these capillaries wide lumina were observed in PBS-injected eyes. The contour of the luminal cell membrane of endothelial cells was smooth and regular, and degenerative changes were not observed. In contrast, in capillaries of the inner nuclear layer in VEGF-injected eyes, the endothelial cells protruded into the capillary lumen resulting in much smaller lumina. The contour of the luminal side of some of these endothelial cells was irregular. In the endothelial cells an enlarged cytoplasm was observed and also their nuclei seemed to be larger. However, the endothelial cells appeared normal, without degenerative changes (exemplified in Figure 4).

Morphometric analysis of electron micrographs of cross sections of capillaries
in the inner nuclear layer of VEGF-injected eyes revealed a significant 2-fold increase (P < 0.0001) in the endothelial cell volume compared to the retinal capillaries in the PBS-injected eyes of both monkeys (Figure 5). The capillaries of VEGF-injected eyes showed a 2 to 3-fold decrease (P < 0.0001) in their luminal volume compared to the lumina of capillaries in the PBS-injected eyes (Figure 5).

The mean number of lateral inter-endothelial cell borders as counted in individual capillary cross-sections was similar between PBS- and VEGF-injected eyes (i.e., 2.0 ±0.1 and 1.9 ±0.05, P>0.05).

COMMENT

Repeated injections (6 to 24 VEGF injections, survival time 12 to 78 days) of VEGF-A in monkey eyes lead to widespread capillary non-perfusion of the retina after 12 to 24 days20. In the present study, we demonstrate that in an earlier stage of this model, after 4 VEGF injections and 9 days survival time, capillaries in the inner nuclear layer of the retina show marked hypertrophy of their endothelial cells, resulting in a significant decrease in capillary luminal volume. This luminal narrowing may be the primary mechanism leading to the occlusion of retinal capillaries and capillary non-perfusion as seen in the later stages of this model20.

In previous studies by Tolentino et al.20 it was demonstrated that inactivated human VEGF does not cause an inflammatory or toxic response in the monkey eye. As the dose of VEGF and total number of injections used here (leading to a peak concentration in the vitreous of approximately 170 ng/ml) are lower than used by Tolentino et al.20, an inflammatory or toxic effect as an explanation of the findings is unlikely.

Although this study is limited to results obtained in two monkeys, the observed differences between the VEGF-injected eyes and PBS-injected eyes were consistent, marked and highly significant for both monkeys. The luminal narrowing in VEGF-injected eyes is not likely to be the result of a collapse of the capillaries, since we observed marked hypertrophy of the endothelial cells as a likely cause. Furthermore, both eyes of each monkey were treated identically before fixation, i.e., by perfusion with PBS at a pressure comparable to the in vivo blood pressure. Since there was no significant difference in the number of lateral inter-endothelial cell borders between capillary cross-sections of PBS- or VEGF-injected eyes, our results do not indicate an increase in endothelial cell number in the deep capillary plexus at the time point studied, and suggest that endothelial hypertrophy rather than hyperplasia is responsible for the luminal narrowing of the inner nuclear capillaries at the time point studied.

The results of our study should be interpreted in the context of the literature.
describing this model of VEGF-induced retinopathy, which is characterized by capillary non-perfusion in its late stages (after approximately 12 days and 6 VEGF injections with a high dose)\textsuperscript{20}. In this context, the time point studied (9 days, after 4 VEGF injections with a lower dose) may be crucial for understanding the pathogenesis of this late capillary non-perfusion. Our light microscopic measurements of the total capillary luminal area per millimeter of retina, which allowed for an overall assessment of the deep retinal capillary bed, indicated a 5 to 7-fold reduction in the total capillary volume in the inner nuclear layer. Electron microscopy of individual capillaries demonstrated that endothelial cell hypertrophy, within the confinement of an only slightly enlarged capillary basement membrane, caused this luminal narrowing. This argues against compression by perivascular retinal edema as a cause of capillary non-perfusion, a mechanism which has previously been suggested \textsuperscript{13, 23}.

Tolentino et al.\textsuperscript{20} observed, by fluorescein angiography (in one monkey, after 4 injections of 1.25 |\mu|g and 8 days survival time, and in two monkeys with much longer exposure to VEGF), vessel dilation, tortuosity and venous beading, occurring before the development of capillary non-perfusion. By histopathological studies, they observed dilated vessels with proliferation of endothelial cells in the most inner layers of the retina, indicating endothelial hyperplasia induced by VEGF in these dilated non-capillary vessels. Although these findings may appear contradictory to our results, it should be noted that the clinically observed vessel dilation may occur primarily in the larger vessels of the primary vascular network. Furthermore, these authors studied this model in a more advanced stage of development, by light microscopy only, and did not study the true capillaries in the primary or deep capillary plexus in detail.

This is the first report identifying endothelial cell hypertrophy as a possible cause of retinal capillary non-perfusion. VEGF-induced luminal narrowing is likely to severely impede capillary blood flow, as flow is inversely related to vascular diameter by the fourth power. Based on our findings, we suggest that exogenous VEGF leads to endothelial cell hypertrophy, capillary luminal narrowing, and eventually capillary non-perfusion, leading to a vicious circle of vascular stasis, ischemia, endogenous VEGF production, further luminal narrowing and so on. The highly dilated vessels with endothelial hyperplasia, as observed in the most inner retina in this model\textsuperscript{20}, may well represent dilated arterioles, arteries, venules, veins and shunt vessels.

Our findings may have implications for the insight into the pathogenesis of human retinal disease associated with VEGF over-expression. From our clinical observations we know that in ischemic retinal vein occlusions, which are known to lead to early VEGF production \textsuperscript{24}, initial stasis of the circulation is often followed only later by true capillary non-perfusion on angiography. In DR, small areas with capillary loss, where local VEGF production probably occurs \textsuperscript{17, 19}, tend to enlarge. Capillary angiomas, which produce large amounts of
VEGF, often induce some degree of capillary non-perfusion in the surrounding retina. These clinical observations are suggestive of the vicious circle described above.

In the current view of the pathogenesis of capillary non-perfusion in diabetic retinopathy, leukocyte adhesion is considered the most important mechanism. Leukocytes may become trapped in retinal capillaries under conditions of reduced perfusion or in the presence of elevated adhesion stress between leukocytes and endothelium. In DM, increased adhesion of leukocytes may result from release of chemotactic factors or expression of adhesion molecules on leukocytes and endothelial cells. When this occurs, even a normal perfusion pressure may fail to dislodge the leukocyte. This mechanism was previously suggested to lead to the irreversible microvascular occlusions in DR.

Leukocyte adhesion was also suggested to cause capillary non-perfusion in experimental VEGF-induced retinopathy. However, in our study, no leukocytes were found in the perfused capillaries, suggesting that in the early stages of this model, no or only few leukocytes are trapped in the capillary bed. As the perfusion pressure used in our experimental setup can be compared to blood pressure in vivo, the perfusion with PBS is unlikely to have washed away trapped leukocytes. Therefore, in this model of VEGF-induced capillary non-perfusion, leukocyte adhesion and trapping may be a secondary phenomenon, not necessarily contributing to the pathogenesis of capillary non-perfusion.

In diabetic retinopathy, leukocyte trapping, endothelial cell hypertrophy and basement membrane thickening may together lead to capillary non-perfusion. VEGF-induced endothelial cell hypertrophy within the confinement of a thickened basement membrane will lead to reduced capillary flow. Furthermore, in diabetic patients, increased numbers of activated, less deformable neutrophils, which release VEGF, are present. These activated neutrophils might not be able to pass successfully through capillaries with decreased diameters, leading to further ischemia, VEGF production and so on.

REFERENCES


