Endothelial Cell Hypertrophy Induced by Vascular Endothelial Growth Factor in the Retina

New Insights Into the Pathogenesis of Capillary Nonperfusion

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Objective: To investigate the mechanism leading to capillary nonperfusion of the retina in a monkey model of vascular endothelial growth factor A (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 of VEGF in one eye and of phosphate-buffered saline in the other eye and were killed at day 9. After perfusion and enucleation, retinal samples were snap frozen for immunohistochemical analysis with the panendothelial cell marker CD31 or were 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 overexpression.

Clinical Relevance: Capillary nonperfusion occurs in diabetic retinopathy and other ischemic diseases associated with overexpression of VEGF. In addition, VEGF-induced endothelial cell hypertrophy may be causative for capillary closure in these diseases.


Capillary nonperfusion 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 nonperfusion 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 nonperfusion 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 nonperfusion 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 up-regulation of intercellular adhesion molecule 1 and leukostasis, supporting a possible role of leukocyte adhesion as a mechanism of VEGF-induced capillary nonperfusion.
MATERIALS AND METHODS

VEGF-INDUCED RETINOPATHY IN MONKEYS

Two cynomolgus monkeys (Macaca fascicularis), a 15-year-old male and a 3-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 and in accordance with the guidelines established for animal care at the University of Nijmegen, the Netherlands.

The animals received 4 injections with phosphate-buffered saline (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, and 7) and 4 injections with bioactive human recombinant VEGF (Harbor Bio-Products, Norwood, Mass) (0.5 µg in 50 µL of PBS) in the right eye (days 0, 2, 4, and 7). Before intravitreal injection, 20 mg/kg of ketamine hydrochloride, 0.005 mg/kg of acepromazine maleate, and 0.03 mg/kg of atropine sulfate were 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 to 80 mm Hg and the eyes were enucleated. The eyes were dissected and samples of retina were either snap frozen in liquid nitrogen for immunohistochemical analysis 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.

RESULTS

LIGHT MICROSCOPIC EVALUATION OF RETINAL CAPILLARIES

Immunohistochemical staining for the panendothelial 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 compared with capillaries in the PBS-injected eyes (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 compared with the PBS-injected eyes (Figure 2).

This was further analyzed by morphometric measurements of the deep capillary plexus of the inner nuclear layer. This retinal layer was chosen because it contains a relatively homogeneous population of capillaries that 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 millimeter of retina) was significantly decreased by 5- to 7-fold (P<.01) when compared with the capillary luminal area per millimeter of 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 (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<.001) in the endothelial cell volume com-

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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, since 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% osmium tetroxide, dehydrated in a series of graded ethanols (3 times for 5 minutes each), and finally embedded in epoxy resin. To calculate the capillary luminal area per millimeter of retina, the following procedure was followed. Six semithin (1-µm) sections (>0.2 mm from each other) of 2 different retina samples of the posterior segment of each eye were cut, stained with toluidine 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, since 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 with an electron microscope (Philips EM201; 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 lumina of the endothelial cells were counted according to 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 interendothelial cell borders was determined in each individual capillary to estimate the number of endothelial cells.

STATISTICAL ANALYSIS

The means (±SEMs) of the calculated total area of capillary lumen per millimeter of retina (of 6 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 endothelial cell borders per capillary were calculated. The Mann-Whitney U test was used to determine the significance of all measurements between PBS- and VEGF-injected eyes of each monkey. The level of significance was set at .05.

Pared with 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<.001) in their luminal volume compared with the lumina of capillaries in the PBS-injected eyes (Figure 5).

The mean number of lateral interendothelial cell borders as counted in individual capillary cross sections was similar between PBS- and VEGF-injected eyes (ie, 2.0±0.1 and 1.9±0.05; P=.47).

COMMENT

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

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

Although this study is limited to results obtained in 2 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, ie, 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 interendothelial 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 nonperfusion in its late stages (after approximately 12 days and 6 VEGF injections with a high dose). 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 nonperfusion.


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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 nonperfusion, a mechanism that has previously been suggested.11,21 Tolentino et al18 observed, by fluorescein angiography (in 1 monkey, after 4 injections of 1.25 µg and a survival time of 8 days, and in 2 monkeys with much longer exposure to VEGF), vessel dilation, tortuosity, and venous beading that occurred before the development of capillary nonperfusion. By histopathologic 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 noncapillary 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, to our knowledge, to identify endothelial cell hypertrophy as a possible cause of retinal capillary nonperfusion. The VEGF-induced luminal narrowing is likely to severely impede capillary blood flow, since 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 eventu-
ally capillary nonperfusion, causing a vicious circle of vascular stasis, ischemia, endogenous VEGF production, and further luminal narrowing. The highly dilated vessels with endothelial hyperplasia, as observed in the most inner retina in this model, 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 overexpression. From our clinical observations, we know that in ischemic retinal vein occlusions, which are known to lead to early VEGF production, initial stasis of the circulation is often followed only later by true capillary nonperfusion on angiography. In DR, small areas with capillary loss, where local VEGF production probably occurs, tend to enlarge. Capillary angiomas, which produce large amounts of VEGF, often induce some degree of capillary nonperfusion in the surrounding retina. These clinical observations are suggestive of the vicious circle described herein.

In the current view of the pathogenesis of capillary nonperfusion in DR, 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 diabetes mellitus, increased adhesion of leukocytes may result from release of chemotactic factors or expression

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**Figure 4.** Electron micrographs of capillaries in the inner nuclear layer of the retina in the phosphate-buffered saline–injected monkey eye (A, B), and vascular endothelial growth factor A (VEGF)–injected monkey eye (C, D). Note the small lumina (L) (arrows) and endothelial cell (E) hypertrophy in the retinal capillaries of the VEGF-injected eye (original magnification ×15000).
of adhesion molecules on leukocytes and endothelial cells.\textsuperscript{7,10,12,13} 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.\textsuperscript{11,17,26}

Leukocyte adhesion was also suggested to cause capillary nonperfusion in experimental VEGF-induced retinopathy.\textsuperscript{10,26} However, in our study, no leukocytes were found in the perfused capillaries, suggesting that in the early stages of this model no or only a few leukocytes are trapped in the capillary bed. Because the perfusion pressure used in our experimental setup can be compared with blood pressure in vivo, the perfusion with PBS is unlikely to have washed away trapped leukocytes. Therefore, in this model of VEGF-induced capillary nonperfusion, leukocyte adhesion and trapping may be a secondary phenomenon, not necessarily contributing to the pathogenesis of capillary nonperfusion.

In DR, leukocyte trapping, endothelial cell hypertrophy, and basement membrane thickening may together lead to capillary nonperfusion. The VEGF-induced endothelial cell hypertrophy within the confinement of a thickened basement membrane will lead to reduced capillary flow. Furthermore, in patients with diabetes, increased numbers of activated, less deformable neutrophils, which release VEGF, are present.\textsuperscript{11,29} These activated neutrophils might not be able to pass successfully through capillaries with decreased diameters, leading to further ischemia and VEGF production.

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