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Aqueous Vascular Endothelial Growth Factor Increases in Anterior Segment Ischemia in Rabbits

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Abstract: Both long posterior ciliary arteries were occluded or the three extraocular muscles were tenotomized to produce anterior segment ischemia in rabbits, and the aqueous levels of vascular endothelial growth factor (VEGF) were measured. The occlusion of both long posterior ciliary arteries led to clinical and histological anterior segment ischemia in varying degrees. The aqueous VEGF levels increased significantly compared with controls on all days examined (Mann-Whitney *U* test: day 1, $P = 0.0039$; day 4, $P = 0.0065$; day 7, $P = 0.0039$; day 14, $P = 0.0104$), while the levels at days 7 and 14 decreased significantly compared with those at day 4 (Wilcoxon signed-rank test; day 4 to day 7 and day 4 to day 14, $P = 0.0464$). In contrast, tenotomy of the three extraocular muscles resulted in no histological changes. The VEGF levels increased significantly compared with controls at day 1 and day 4 after surgery (Mann-Whitney *U* test: day 1 and day 4, $P = 0.0104$), while the levels at day 14 decreased significantly compared with those at day 1 (Wilcoxon signed-rank test, $P = 0.0499$). Aqueous VEGF levels represent the severity of anterior segment ischemia and could be used as an indicator for the extent of ischemia. **Jpn J Ophthalmol 1998;42:85–89** © 1998 Japanese Ophthalmological Society

Key Words: Anterior segment ischemia, rabbit, strabismus surgery, vascular endothelial growth factor (VEGF).

Introduction

Anterior segment ischemia or necrosis is, although rare, a well-known complication of surgery for retinal detachment[1] and strabismus.[2,3] The development of anterior segment ischemia has been reported in patients who underwent surgery involving three or four rectus muscles. Surgery involving multiple rectus muscles at one session or with a short-term interval could impair the anterior ciliary arteries that provide the major blood supply for the anterior segment of the eye.

Rabbit eyes have a unique circulatory architecture in the anterior segment where the major iridic circle is supplied only by both long posterior ciliary arteries, in contrast with primates and humans with such collaterals as anterior ciliary arteries directly anastomosing to the long posterior ciliary arteries.[4] Therefore, the occlusion of both long posterior ciliary arteries in rabbits resulted in severe anterior segment necrosis.[5–8] In contrast, anterior ciliary arteries (also called muscular arteries) at the superior and inferior rectus and inferior oblique muscles in rabbits flow into a series of perilimbal vascular arcades that supply blood only to the superficial region of the anterior segment.[9]

Vascular endothelial growth factor (VEGF) is a mitogen specific to vascular endothelial cells, and its secretion increases during hypoxia,[10–12] calling into question whether VEGF plays a role in anterior segment ischemia. In this study, we measured the concentration of VEGF in the aqueous after inducing ischemia by diathermal occlusion of both long posterior ciliary arteries or by tenotomy of the three extraocular muscles in rabbits. We compared the clinical and histological severity of ischemic changes from the viewpoint of aqueous VEGF levels after these two procedures.

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Materials and Methods

Animal Experiments

Twelve adult pigmented male rabbits, weighing 1.8 to 3.2 kg, were anesthetized with intravenous pentobarbital at a dose of 50 mg per kg of body weight. Experimental eyes were the left ones in each rabbit, and the right eyes served as controls. The experimental eyes were divided into the following two groups of procedures: occlusion of both long posterior ciliary arteries versus tenotomy of superior and inferior rectus and inferior oblique muscles. Conjunctival limbal peritomy was performed to expose both long posterior ciliary arteries. A diathermy unit was used to cauterize directly the long posterior ciliary arteries along their entire visible course. The conjunctiva was pulled back to the limbus without suture. In contrast, insertions of the superior and inferior rectus and inferior oblique muscles were exposed after conjunctival limbal peritomy and sectioned without diathermy for hemostasis. An antibiotic ointment was instilled into the eyes. The control eyes underwent only conjunctival limbal peritomy.

Aqueous samples were obtained from each experimental and control eye before surgery and 1, 4, 7, and 14 days after surgery, using a 30-gauge needle attached to a siliconized 1-mL syringe passed through the limbus under an operating microscope. Samples were transferred to sterile tubes and frozen at −20°C until use. Animals were sacrificed 14 days after the surgery. The enucleated eyes were fixed with 3.7% formaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) overnight and embedded in paraffin. Sections were cut and stained with hematoxylin-eosin. All animal studies were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Measurement of VEGF

VEGF concentrations in aqueous samples were determined in duplicate by a VEGF assay kit (Quantkine™, R&D Systems, Minneapolis, MN, USA) based on a quantitative sandwich enzyme-linked immunosorbent assay technique. A series of standards and samples, diluted twofold or fivefold with buffer, were incubated for 2 hours at room temperature in wells of a microtiter plate coated with monoclonal antibody against human $VEGF_{165}$. After washing the wells with the buffer provided, horseradish peroxidase-conjugated polyclonal antibody against VEGF was added to the wells to sandwich the VEGF immobilized during the first incubation. Following a wash, substrate solution was added to the wells and color was developed. The intensity of the color was measured at 450 nm by a microphotometer (EIA Reader, Model 2550, Bio-Rad Laboratories Japan, Tokyo) and compared with the standard curve, which showed linearity with the concentration of VEGF. The concentrations of the samples were determined from an average of duplicate measurements.

Results

Occlusion of Both Long Posterior Ciliary Arteries

Corneal diffuse edema and new vessels invading from the limbus toward its center were observed clinically in varying degrees in all experimental rabbit eyes (Figure 1). Figure 2 shows temporal changes in aqueous VEGF concentrations after the experimentally induced anterior segment ischemia. The VEGF concentrations in the aqueous of experimental eyes were 54.0–271.0 pg/mL (median = 155.8 pg/mL) preoperatively; 1166.2–8266.0 pg/mL (median = 4525.5 pg/mL) at day 1, 833.0–31425.0 pg/mL (median = 9689.3 pg/mL) at day 4, 921.0–28650.0 pg/mL (median = 4122.2 pg/mL) at day 7, and 457.0–11005.0 pg/mL (median = 1515.9 pg/mL) at day 14. In contrast, VEGF concentrations in the aqueous of control eyes were 63.0–262.0 pg/mL (median = 158.4 pg/mL) preoperatively, 98.1–350.0 pg/mL (median = 153.7 pg/mL) at day 1, 56.0–1280.0 pg/mL (median = 184.1 pg/mL) at day 4, 6.0–174.0 pg/mL (median = 146.6 pg/mL) at day 7, and 137.0–918.0 pg/mL (median = 171.0 pg/mL) at day 14. There were no signif-

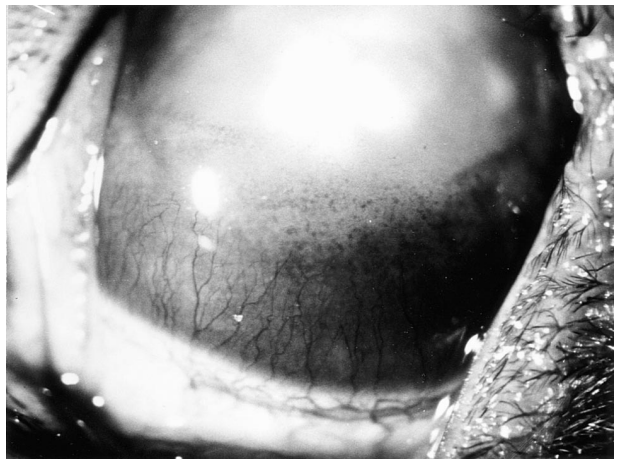

Figure 1. Corneal edema and neovascularization 14 days after occlusion of both long posterior ciliary arteries in left eye of rabbit.

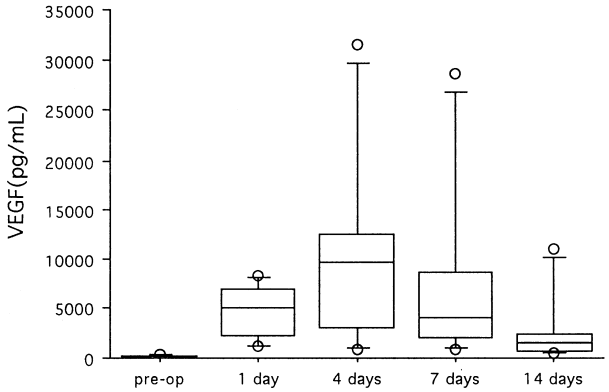

Figure 2. Box-and-whisker plot for changes in aqueous vascular endothelial growth factor (VEGF) levels in eyes with occlusion of both long posterior ciliary arteries. Middle line = median; upper and lower sides of box = 75% and 25% quartile values; upper and lower I-bars = 90% and 10% limits.

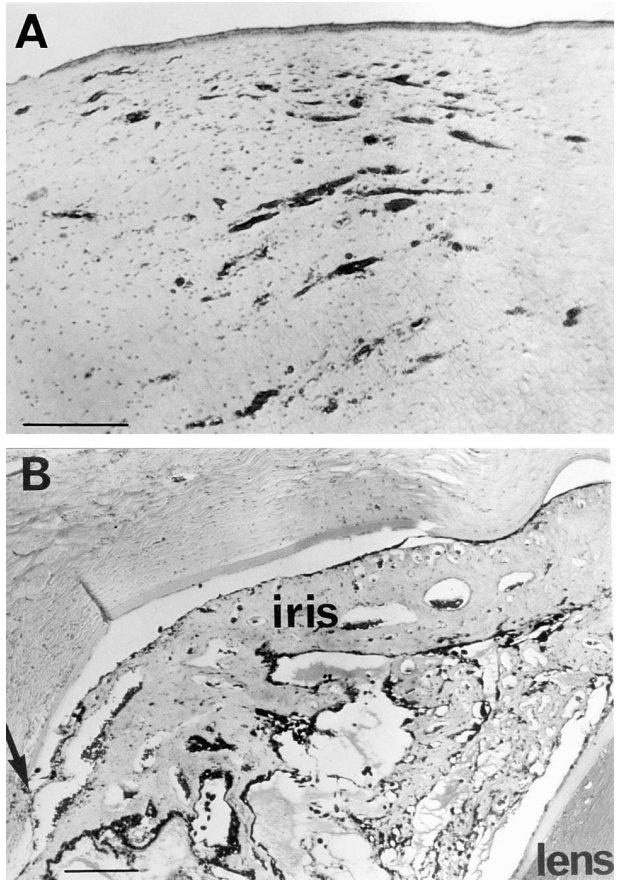

Figure 3. Histological changes 14 days after occlusion of both long posterior ciliary arteries. Note corneal neovascularization (**A**) and massive necrosis of the iris and ciliary processes (**B**). Inflammatory cells are absent. Angle is indicated by arrow. Hematoxylin-eosin stain. Bar = 200 μm.

icant changes in aqueous VEGF levels in control eyes (Wilcoxon signed-rank test, preoperatively to each postoperative day, $P > 0.29$). The VEGF concentrations in the experimental eyes increased significantly compared with those in the control eyes on all days tested (Mann-Whitney U test: day 1, $P = 0.0039$; day 4, $P = 0.0065$; day 7, $P = 0.0039$; day 14, $P = 0.0104$). In the experimental eyes, VEGF levels at days 7 and 14 decreased significantly compared with those at day 4 (Wilcoxon signed-rank test, day 4 to day 7 and day 4 to day 14, $P = 0.0464$). Overall, aqueous VEGF concentrations in the experimental anterior segment ischemia increased during the initial 4 days and then decreased gradually.

Histologic examinations of the eyes enucleated at day 14 disclosed severe necrosis of the anterior segments in varying degrees. In the severe cases, the cornea showed marked stromal edema, and neovascularization was found at the subepithelial level to the middle stroma. The iris and ciliary processes had no cellular details and showed hyaline degeneration, whereas inflammatory cells were absent (Figure 3). Histological severity of ischemic necrosis, varying from rabbit to rabbit, coincided with clinical presentations and had a positive relationship with the aqueous VEGF levels (Table 1). The posterior segment did not have ischemic changes.

Tenotomy of the Three Extraocular Muscles

Moderate corneal diffuse edema with no neovascularization was observed clinically in all the rabbits. Figure 4 shows temporal changes in aqueous VEGF concentrations after the transection of the three extraocular muscles. The VEGF concentrations in the aqueous of experimental eyes were 100.8–263.8 pg/mL (median = 103.2 pg/mL) preoperatively, 173.2–400.9 pg/mL (median = 245.2 pg/mL) at day 1, 135.4–411.9 pg/mL (median = 252.2 pg/mL) at day 4, 47.0–428.3 pg/mL (median = 112.5 pg/mL) at day 7, and 2.0–254.8 pg/mL (median = 172.8 pg/mL) at day 14. In contrast, VEGF concentrations in the aqueous of control eyes were 61.5–258.2 pg/mL (median = 162.4 pg/mL) preoperatively, 56.4–181.4 pg/mL (median = 107.4 pg/mL) at day 1, 92.8–127.4 pg/mL (median = 110.1 pg/mL) at day 4, 43.6–184.1 pg/mL (median = 130.1 pg/mL) at day 7, and 41.0–154.3 pg/mL (median = 119.4 pg/mL) at day 14. The VEGF concentrations in the experimental eyes increased significantly compared with those in the control eyes at day 1 and day 4 after the operation

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Table 1. Histological Findings and Aqueous Vascular Endothelial Growth Factor (VEGF) Levels in Eyes Wth Occlusion of Both Long Posterior Ciliary Arteries

Rabbit No.	Cornea	Ciliary Body			Iris			VEGF (pg/mL) at day 14
	New Vessels	Nonpigment Epithelial Necrosis	Pigment Epithelial Necrosis	Stromal Necrosis	Epithelial Necrosis	New Vessels	Stromal Necrosis	
1	Yes	No	No	No	No	Yes	No	1235.0
2	Yes	No	No	No	No	Yes	No	457.0
3	Yes	Yes	Yes	Yes	Yes	Yes	Yes	11005.0
4	Yes	Yes	Yes	No	Yes	Yes	No	1796.7
5	Yes	Yes	Yes	No	Yes	Yes	Yes	660.3

Epithelial necrosis indicates pyknosis or karyorrhexis, while stromal necrosis indicates no cellular details or hyaline degeneration. Histological severity of ischemic necrosis shows a positive relationship with the aqueous VEGF levels.

(Mann–Whitney U test, day 1 and day 4, $P = 0.0104$). In the experimental eyes, the VEGF levels at day 14 decreased significantly compared with those at day 1 (Wilcoxon signed-rank test, $P = 0.0499$). Overall, high levels of aqueous VEGF in the eyes with disinsertion of the three extraocular muscles lasted for the initial 4 days.

Histological examination of the eyes enucleated at day 14 showed no apparent changes in the anterior segments as well as in the posterior segments.

Discussion

The clinical and histological manifestations of the anterior segment ischemia after the occlusion of both long posterior ciliary arteries in this study were the same as previously reported.[5–8] The severity of anterior segment necrosis, varying from rabbit to rabbit, could be attributed to variant circulatory architectures in individual rabbits or to technical variables in diathermy. The major iridic circle supplied by the long posterior ciliary arteries in rabbits receives small, variable anastomoses from the perilimbal vascular arcades supplied by the anterior ciliary arteries. These anastomoses would contribute to varying manifestations of ischemia after the occlusion of the long posterior ciliary arteries.

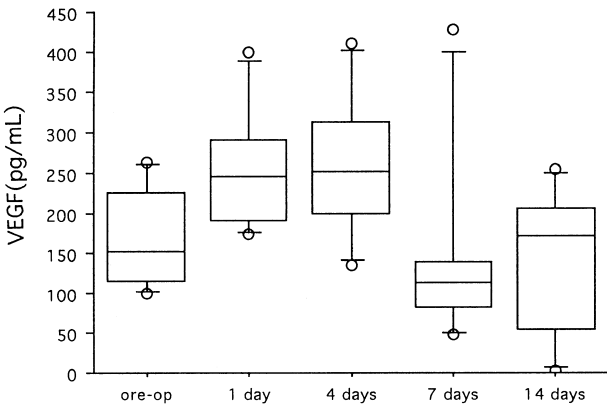

Figure 4. Box-and-whisker plots for changes in aqueous vascular endothelial growth factor (VEGF) levels in eyes with tenotomy of superior and inferior rectus and inferior oblique muscles. Middle line = median; upper and lower sides of box = 75% and 25% quartile values; upper and lower I-bars = 90% and 10% limits.

The effect of multiple paracenteses to obtain aqueous samples successively was negligible since no rise in aqueous VEGF levels was observed in control eyes. The aqueous VEGF increased to the highest level as soon as the anterior segment fell into ischemia by the occlusion of both long posterior ciliary arteries. Furthermore, the levels of aqueous VEGF were correlated with histological and clinical ischemic changes; namely, the more severe the histological and clinical ischemic findings were, the higher the VEGF levels. These results suggest that anterior segment ischemia causes an elevated aqueous VEGF level in accordance with the ischemic severity. Therefore, the aqueous VEGF is a parameter for the extent of anterior segment ischemia.

Arteries from the superior and inferior rectus and inferior oblique muscles in rabbit eyes extend forward along the muscle insertions to the episcleral tissues in the limbal region but do not supply direct branches to the major iridic circle[9] as in human eyes. Therefore, it has been contended that rabbit eyes are not a suitable model for anterior segment ischemia secondary to tenotomy of extraocular muscles, which is observed after surgery for retinal detachment and strabismus in human eyes. In the present study, tenotomy of the three extraocular muscles led

to an increase of the aqueous VEGF, although in a small quantity, lasting for only 4 days, and was accompanied by no histological changes 14 days after the surgery. These facts indicate that the tenotomy of the three extraocular muscles caused reversible anterior segment ischemia at a subhistological level. The VEGF would play a role in healing the ischemia by restructuring damaged vessels, and subsequent re-establishment of normal circulation would, in turn, depress the production of VEGF.

The VEGF would be produced by cells in the anterior segment in response to its ischemia. The aqueous VEGF levels returned to normal after 4 days in the mild, reversible ischemia observed in some eyes with both long posterior ciliary arterial occlusions and in all the eyes with disinsertion of the three extraocular muscles. The VEGF levels remained high in severe irreversible ischemia in some eyes caused by occlusion of the long posterior ciliary arteries. Severe ischemia resulted in extensive necrosis of the anterior segment, which naturally caused death of VEGF-producing cells. The decrease in aqueous VEGF in severe ischemia at day 14 could be attributed in part to the death of these cells.

In conclusion, the occlusion of both long posterior ciliary arteries caused severe irreversible necrosis of the anterior segment in rabbits, while tenotomy of the three extraocular muscles with the anterior ciliary arteries resulted in mild, reversible ischemic changes. The aqueous VEGF levels represented the severity of these ischemic changes in rabbits and, therefore, could be used as a parameter to assess the extent of anterior segment ischemia and its prognosis after surgery for retinal detachment and strabismus in human eyes.

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