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LABORATORY INVESTIGATIONS

Aqueous Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor Decrease During Regression of Rabbit Pupillary Membrane

Toshihiro Yanagawa, Toshihiko Matsuo and Nobuhiko Matsuo

Department of Ophthalmology, Okayama University Medical School, Okayama City, Japan

Abstract: We studied the roles of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in the aqueous during regression of the pupillary membrane in rabbits. Aqueous samples were obtained using a 30-gauge needle passed through the limbus in newborn rabbits. The VEGF and bFGF levels were measured by a quantitative sandwich enzyme-linked immunosorbent assay. The aqueous VEGF levels at 60 days of age (29.0–351.9 with a median of 190.5 pg/mL) decreased significantly compared with those at 12 and 20 days of age (356.7–1148.3 with a median of 752.5 pg/mL at 12 days of age and 193.5–657.7 with a median of 425.6 pg/mL at 20 days of age, Mann-Whitney U Test, $P < 0.0001$ and $P = 0.002$, respectively). The aqueous bFGF levels at 60 days of age (0.0–126.2 with a median of 63.1 pg/mL) decreased significantly compared with those at 12 days of age (33.4–301.3 with a median of 167.4 pg/mL, $P < 0.0001$). Light microscopically, the pupillary membrane at 12 days of age was rich with capillaries that were subsequently closed at 20 days of age, and the membrane itself disappeared at 60 days of age. The aqueous VEGF and bFGF levels decreased in the process of regression of the pupillary membrane, suggesting that VEGF or bFGF played a role in eye development such as maintenance of the pupillary membrane. **Jpn J Ophthalmol 1998;42:157–161**

Key Words: Apoptosis, basic fibroblast growth factor, capillaries, eye development, pupillary membrane, rabbit, vascular endothelial growth factor.

Introduction

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are candidates for angiogenic factors in vivo and in vitro.[1–5] Vascular endothelial growth factor has a signal peptide allowing its secretory release from cells,[6] but bFGF lacks this signal peptide for secretion.[7] Unlike bFGF, which is mitogenic for many types of cells, VEGF is specific for vascular endothelial cells in vivo,[8] and its production is enhanced by hypoxia.[9] Vascular endothelial growth factor also increases vascular permeability, an activity for which it has been originally called vascular permeability factor.[10]

Vascular endothelial growth factor and bFGF are involved in the development of embryonic blood vessels.[11] Targeted inactivation of flk-1, a receptor of VEGF, in transgenic mice resulted in a phenotype completely lacking both vascular and blood island progenitor cells.[12] Other groups have demonstrated that bFGF is required for the induction of vasculogenesis in embryoid bodies of the quail.[13] Because vasculogenesis occurs spontaneously in mouse embryoid bodies, it is not yet known whether bFGF is absolutely required for vasculogenesis in mammalian systems.[14] However, endodermally derived bFGF stimulates vitilline vasculogenesis through its receptors expressed in the developing yolk sac.[15]

Regression of intraocular vascular tissues like the pupillary membrane and hyaloid vasculature is as important as vasculogenesis for eye development. However, its relationship with growth factors has not yet been studied. The pupillary membrane is a transitory vascular connective tissue covering the anterior surface of the lens during embryonic development. In 12-day-old rabbits, the pupillary membrane

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Address correspondence and reprint requests to: Toshihiro YANAGAWA, MD, Department of Ophthalmology, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama City, Okayama 700, Japan

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and its capillary network can be observed with slit-lamp biomicroscopy. Thereafter, it begins to regress spontaneously, resulting in white strands near the pupil margin at 20 days of age.[16] We investigated the roles of VEGF and bFGF in the aqueous during regression of the pupillary membrane in rabbits.

Materials and Methods

After rabbits were sedated with intramuscular injection of ketamine (50 mg/kg), the pupillary membrane was confirmed with slit-lamp biomicroscopy (Figure 1A, 1B). Primary aqueous samples were obtained from each eye of different sedated animals, using a 30-gauge needle attached to a siliconized 1-mL syringe passed through the limbus under an operating microscope in 13 eyes of albino rabbits at 12 days of age, 4 eyes at 15 days of age, 2 eyes at 17 days of age, 11 eyes at 20 days of age, and 11 eyes at 60 days of age for measurement of VEGF and in 23 eyes at 12 days of age, 11 eyes at 20 days of age, and 14 eyes at 60 days of age for measurement of bFGF. Samples were immediately transferred to sterile tubes and frozen at −20°C until use. Enucleated eyeballs were fixed with 3.7% formaldehyde in 0.1-M phosphate buffer (pH 7.4) overnight and embedded in paraffin. Sections were cut and stained with hematoxylin eosin. All studies were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The VEGF and bFGF levels in the aqueous were measured in duplicate by assay kits (Quantkine, R&D Systems, Minneapolis, MN, USA) based on a quantitative sandwich enzyme-linked immunosorbent assay technique. Standards and samples were incubated for 2 hours at room temperature in the wells of a microtiter plate coated with monoclonal antibody specific for VEGF or bFGF. After washing with the buffer provided, horseradish peroxidase-conjugated polyclonal antibody specific for VEGF or bFGF was added to the wells to sandwich the VEGF or bFGF 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 by a spectrophotometer set at 450 nm (EIA Reader, Model 2550, Bio-Rad Laboratories Japan, Tokyo) and compared with the standard curve, which showed linearity with the concentration. Thus, VEGF and bFGF levels in the samples were determined from an average of duplicate measurements.

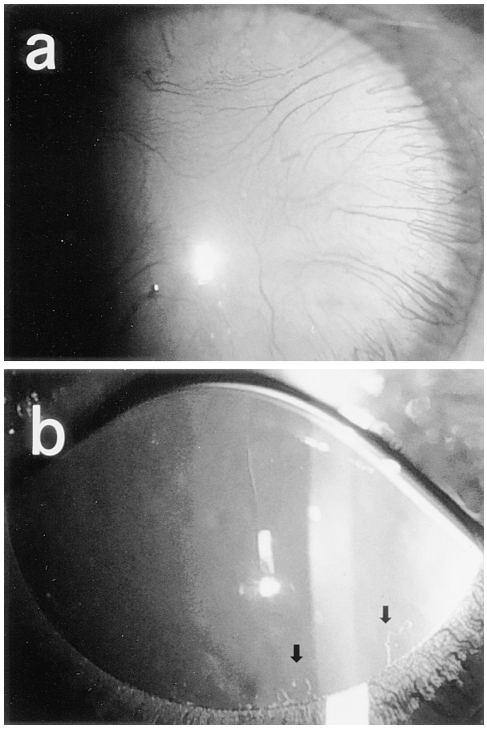

Figure 1. The pupillary membrane in a 12-day-old rabbit (**A**) and a 20-day-old rabbit (**B**). Note rich capillaries (**A**) and white strands (arrows in **B**).

Results

Aqueous samples could be obtained in 20 to 40 μL from each eye. Well-correlating standard curves were obtained for VEGF and bFGF ($R^2 = 0.975$ and 0.992, respectively). Figure 2 shows the aqueous VEGF and bFGF levels during regression of the pupillary membrane. The VEGF levels in the aqueous were 356.7–1148.3 pg/mL (median = 752.5 pg/mL) at 12 days of age, 193.5–657.7 pg/mL (median = 425.6 pg/mL) at 20 days of age, and 29.0–351.9 pg/mL (median = 190.5 pg/mL) at 60 days of age. The VEGF levels at 60 days of age decreased significantly compared with those at 12 and 20 days of age (Mann-Whitney *U* Test, $P < 0.0001$ and $P = 0.002$, respectively). The aqueous bFGF levels were 33.4–301.3 pg/mL (median = 167.4 pg/mL) at 12 days of age, 1.8–254.1 pg/mL (median = 128.0 pg/mL) at 20 days of age, and 0.0–126.2 pg/mL (median = 63.1 pg/mL) at 60 days of age. The bFGF levels at 60 days of age decreased significantly compared with those at 12 days of age (Mann-Whitney *U* Test, $P < 0.0001$). Overall, there was a tendency for VEGF and bFGF levels to decrease gradually during regression of the pupillary membrane.

Light microscopically, the pupillary membrane, located in front of the lens and linked to the iris in 12-

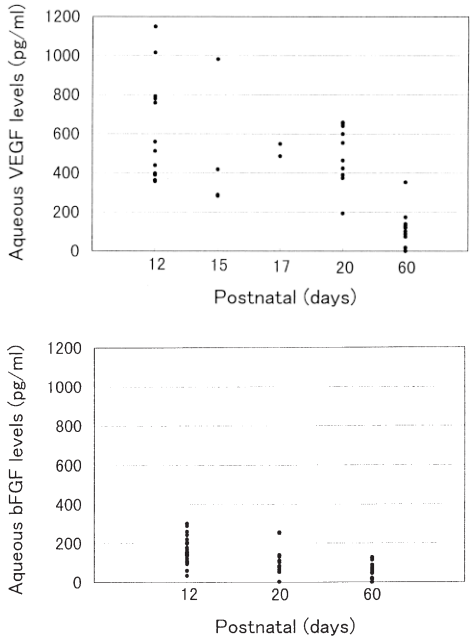

Figure 2. The aqueous vascular endothelial growth factor (VEGF) (**top**) and basic fibroblast growth factor (bFGF) (**bottom**) levels during regression of the pupillary membrane. Both growth factors decrease during the regression.

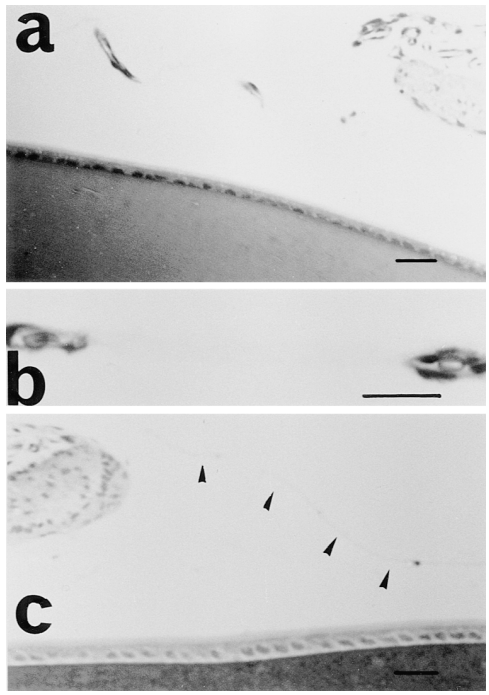

Figure 3. Light microscopy of the pupillary membrane in a 12-day-old rabbit (**A**, **B**: higher magnification of **A**) and a 20-day-old rabbit (**C**). Note a membrane with clear endothelial nuclei and a lumen filled with red blood cells (**B**) located in front of the lens and linked to the iris (**A**). Vascular endothelial nuclei and red blood cells disappear in a thin pupillary membrane (arrowheads in **C**). Bar = 10 μm.

day-old rabbits, had many capillaries with red blood cells in their lumens and with endothelial nuclei visualized clearly (Figure 3A, 3B). The pupillary membrane at 20 days of age, still covering the pupil, lost vascular endothelial nuclei and became a white strand (Figure 3C). At 60 days of age, the pupillary membrane could not be observed. The hyaloid vasculature and the tunica vasculosa lentis had already disappeared by as early as 12 days of age.

Discussion

The present findings demonstrate that the aqueous VEGF and bFGF levels in 12-day-old rabbits with functional vessels in the pupillary membrane were higher than those in 20-day-old ones with no functional vessels, suggesting a causal relationship between the aqueous VEGF and bFGF levels and pupillary membrane regression. However, this study could not necessarily establish the direct relationship between these growth factors and the pupillary membrane, because there may be other unknown factors involved in the process of pupillary membrane regression. In addition, other embryonal vascular systems in the eye, such as the hyaloid vasculature and the tunica vasculosa lentis, might underlie the levels of these growth factors. The hyaloid vasculature and the tunica vasculosa lentis have already regressed by as early as 12 days of age when the aqueous levels of VEGF and bFGF still remain high. The high levels of the growth factors might otherwise be residual after these embryonal vascular systems have regressed.

The aqueous VEGF level in 12-day-old rabbits is almost half its aqueous level in a primate model for central retinal vein occlusion (about 1600 pg/mL at the peak level)[17] and half its vitreous level in human eyes with proliferative diabetic retinopathy (median of 1164 pg/mL).[18] In contrast, the aqueous bFGF level in those rabbits is much lower than its vitreous level in human eyes with proliferative diabetic retinopathy (more than 30 ng/mL).[19] The meaning of these facts remains unclear at the moment but may reflect that VEGF plays a more central role than bFGF in eye development.

In this study, the aqueous VEGF levels varied widely from rabbit to rabbit. Although the pupillary membrane is regressing, the aqueous VEGF levels would decrease day by day, even hour by hour. The

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rabbits we used were born on the same day but at different times. These individual differences might underlie the aqueous VEGF levels in a wide range.

One possible explanation for the relationship between eye development and decreased levels of VEGF and bFGF is that the aqueous VEGF and bFGF, if produced by vascular endothelial cells in embryonal vascular systems in the eye including the pupillary membrane as by other vascular endothelial cells,[5,20,21] would naturally decrease as the embryonal vascular systems regress. The second possibility for a more active role of VEGF goes as follows: 12-day-old rabbits might have hypoxia in the anterior chamber, which is shallow and has a strong demand of oxygen for the developing ciliary processes or the lens. This hypoxic state would lead to increased levels of VEGF in the anterior chamber. In contrast, the anterior chamber at 20 days of age becomes deeper, when the ciliary processes and the lens have developed enough so that they do not demand much oxygen, which would result in the decreased aqueous levels of VEGF, leading finally to the regression of the pupillary membrane and other embryonal vascular systems in the eye. The aqueous bFGF would not play this role because the production of bFGF is not up regulated by hypoxia in contrast with VEGF.[22] However, the combined effects of VEGF and bFGF during the regression of the pupillary membrane could not be excluded, as these two growth factors have a synergistic effect on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels in vitro.[23]

One feature common to most examples of tissue regression is the involvement of macrophages. Macrophages can be observed around the pyknotic nuclei of redundant neurons of the retina in the mouse,[24] around the debris from dying cells in the posterior necrotic zone of the developing chick wing,[25] and around the apoptotic bodies from the regressing intestinal epithelium of the tadpole.[26] During the regression of the pupillary membrane, macrophages also seem to play an important role in extinction of vascular endothelial cells. Matsuo and Smelser observed macrophages, appearing on the surface of the white strand in the pupillary membrane and within the lumen of its vessels, and suggested that macrophages were responsible for killing vascular endothelial cells.[16] More recently, Lang and Bishop demonstrated that the absence of functional macrophages in transgenic mice resulted in a lack of programmed cell death and abnormal tissue remodeling in the eye, leading to the persistent presence of both the hyaloid vasculature and the pupillary membrane.[27] They also showed that the death of vascular endothelial cells, occurring during the regression of the pupillary membrane in rats, had all the characteristics of apoptosis and suggested that macrophages induced apoptosis of vascular endothelial cells during its regression.[28] However, a trigger for the apoptosis, and how macrophages induce the apoptosis, remains unknown.

Recently, high levels of VEGF or bFGF have been reported to suppress apoptosis of vascular endothelial cells in vitro,[29–32] although the mechanism remains unclear. The present findings, together with the previous observations,[16,28] suggest that VEGF and bFGF prevent macrophages from inducing apoptosis of vascular endothelial cells in the pupillary membrane. Further studies are needed to elucidate whether and how VEGF and bFGF are related to such a process of eye development as pupillary membrane regression.

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