Iris movement mediates vascular apoptosis during rat pupillary membrane regression

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IN THE COURSE OF MAMMALIAN eye development, the pupillary membrane (PM) and hyaloid vessels, including the hyaloid artery and the tunica vasculosa lentis, nourish the immature lens, retina, and vitreous (7). These vessels are known to regress during the later stages of ocular development, prenatally in humans and within the first few weeks of postnatal development in rodents. The PMs of newborn rats, which arise from the vessels of the iris stroma and cover the anterior surface of the lens, have been utilized as a model system for studying the mechanism underlying vascular regression during development (15, 16, 20, 28). To date, apoptosis has been implicated in the process of PM regression (15); however, little is known of the mechanism that governs the initiation of apoptosis during this process.

In the fully developed eye, constrictive and dilative iris movements, that is, the autonomic responses of miosis and mydriasis in the iris, function to control the amount of light that reaches the lens, reduce optical aberrations, and improve the depth of focus (11). The iris has also been shown to constrict and dilate during the developmental period before the eyelids open and light is admitted into the eye (4). However, its function during this period remains unknown.

In the current study, we initially observed that the regression of the PM and the development of iris motility were temporally associated, and blood flow within the PM changed dramatically in conjunction with iris movement. On the basis of these observations, we hypothesized that iris movement induces the regression of the PM by changing the blood flow within it. The objectives of this study were, therefore, to determine the causal relationship between iris movement and PM regression and to define the function of the iris during the developmental period.

MATERIALS AND METHODS

Animals. All of the animal experiments were carried out in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research by the Association for Research in Vision and Ophthalmology, and were approved by the Animal Care and Use Committee of Okayama University, Japan. We bred Wistar and Brown Norway neonatal rats [body weight at postnatal day 3 were 6–9 g (SLC, Hamamatsu, Japan)] at 23°C under a 12:12-h light-dark cycle. We anesthetized the rats with an intramuscular injection of ketamine (Sankyo, Tokyo, Japan) and xylazine (Bayer Health Care, Tokyo, Japan) at 1.6 mg/g and 32 mg/g, respectively, as described previously (20). A small incision (~0.5 mm in length) could be made without any bleeding along the line at which the eyelids would eventually open. Each eye drop (~100 μl) was applied into the conjunctival sac through the incision with a 24-gauge elastic needle at 5-min intervals (the number of agents depended on the protocols; details are described below). It is well known that the mydriatic agent applied to one eye provokes a dilative response in the contralateral, untreated eye (23). For this reason, we considered that the contralateral eye is not appropriate as a control and carried out all experiments unilaterally. Therefore, the numbers (n) presented here for each experiment indicate the number of both eyes and rats.

Time course of the regression of the PM. At postnatal (P) day 3 (P3), P6, P8, P10, P12, and P14, the iris of Wistar rat neonate was

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Fig. 1. In vivo imaging system and experimental protocol for the continuous inhibition of iris movement. A: schematic showing the in vivo imaging system. B: top: the intravital video microscope with a charge-coupled device. Bottom: a magnified view of the objective lens of the video microscope. C: experimental protocol for evaluating the effects of iris movement on the time course of regression of the pupillary membrane (PM), apoptosis, and macrophage migration.

We divided the Wistar rat littermates into two groups and regulated the iris movement from P7 to P12 by applying eye drops every 4 h, both day and night, as follows: group 1 (control) received physiological saline \( (n = 4) \) and group 2 (continuous inhibition of iris movement) received 1% atropine sulfate \( (n = 5) \) (Fig. 1C). For the animals in group 2, the iris movement was continuously inhibited from P0 to P12 by suppressing the constriction of the iris (continuous iris dilatation). Because the neonatal rat iris was constitutively dilated and unresponsive to autonomic stimulation before P8 (see RESULTS and Fig. 2B), the eye drops were only used from P7 to P12. At P12, when the PM has normally regressed by \( \sim 50\% \) (see RESULTS and Fig. 2A), we quantified the number of vascular junctions in the PM using the intravital video microscope.

We conducted the same protocol as described above for another two sets of Wistar rat littermates to investigate the effects of iris movement on apoptosis in the PM and macrophage migration toward the PM. At P12, the PMs were disected as reported previously (15) and used for analyses. One set of littermates was used to evaluate the proportion of apoptotic cells \( (n = 5 \text{ in both group 1 and 2}) \), and the other set was used to evaluate the proportion of macrophages \( (n = 3 \text{ in group 1 and } n = 4 \text{ in group 2}) \). To detect apoptotic cells, the PMs were stained using terminal deoxynucleotidyl dUTP nick-end labeling (TUNEL) with an ApopTag S7110 detection kit (Chemicon International, Temecula, CA), according to the manufacturer’s instructions. Macrophages were detected using the ED-1 antibody (BMA Biomedicals, Augst, Switzerland) and goat anti-mouse immunoglobulin G (Molecular Probes, Eugene, OR), according to the manufacturers’ instructions. All specimens were counterstained with 4,6 diamidino-2-phenyl-indole dihydrochloride (DAPI; Sigma). We observed the specimens under a fluorescence microscope (Axioplan; Carl Zeiss, Heidelberg, Germany) and recorded the images with a charge-coupled device camera (Axiocam; Carl Zeiss). TUNEL-positive cells, which have characteristic condensed chromatin as revealed by DAPI staining, were recognized as apoptotic cells (21). We quantified the number of apoptotic cells, ED-1-positive cells, and DAPI-positive cells, and then calculated the proportions of apoptotic cells and macrophages to DAPI-positive cells.

To study the effect of iris movement on PM regression in pigmented rats, we divided Brown Norway rat littermates into two groups and regulated the iris movement as described above \( (n = 4 \text{ in both groups 1 and 2}) \). In group 2, we inhibited iris movement over a longer time period, from P7 to P56, to investigate the impact of iris movement on PM regression further. At P12 in group 1 and at P56 in group 2, we examined the morphology of the PM using an electron microscope as described below \( (n = 2 \text{ in both group 1 and 2}) \) (15).

Transmission electron microscopy. We fixed the isolated PM with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) using the intravital video microscope and measured the change in the blood flow velocity along with the iris movement as reported previously (22).

Effects of iris movement on PM regression, apoptosis, and macrophage migration. We divided the Wistar rat littermates into two groups and regulated the iris movement from P7 to P12 by applying eye drops every 4 h, both day and night, as follows: group 1 (control) received physiological saline \( (n = 4) \) and group 2 (continuous inhibition of iris movement) received 1% atropine sulfate \( (n = 5) \) (Fig. 1C). For the animals in group 2, the iris movement was continuously inhibited from P0 to P12 by suppressing the constriction of the iris (continuous iris dilatation). Because the neonatal rat iris was constitutively dilated and unresponsive to autonomic stimulation before P8 (see RESULTS and Fig. 2B), the eye drops were only used from P7 to P12. At P12, when the PM has normally regressed by \( \sim 50\% \) (see RESULTS and Fig. 2A), we quantified the number of vascular junctions in the PM using the intravital video microscope.

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Transmission electron microscopy. We fixed the isolated PM with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4)
overnight. We then postfixed the specimens with 1% osmium tetroxide (Merck, Darmstadt, Germany) at 4°C for 1 h. After dehydration with an acetone series (50, 70, 80, 90, 95, and 100%), the specimens were embedded in Epon 812 (Oken Shoji, Tokyo, Japan) and polymerized at 60°C for 3 days. We then cut ultra-thin sections with a diamond knife, and stained them with 5% aqueous uranyl acetate and lead citrate. Finally, we observed the specimens under a JEM 1200EX transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

**Statistical analysis.** We used the Student’s t-test to analyze the capillary density. The Mann-Whitney U-test was used to analyze the proportion of apoptotic cells and macrophages. All numerical data are presented as means \( \pm \) SE. We considered \( P \) values < 0.05 to be statistically significant.

**RESULTS**

Regression of the PM coincided with the development of iris motility. As illustrated in Fig. 2, the PM showed slight regression and the iris barely responded to autonomic stimulation up to P8. However, from P8 to P14, the capillary meshwork rapidly regressed, and the iris became progressively more responsive to both sympathetic and parasympathetic stimulation.

**Constriction and dilation of the iris caused blood flow cessation and resumption within the PM.** Under control conditions, the vascular network of the PM had tetra-radial symmetry and consisted of a two-dimensional array of arterioles (diameter = 21.6 \( \pm \) 1.5 \( \mu \)m, \( n = 10 \) arterioles), venules (diameter = 23.3 \( \pm \) 2.2 \( \mu \)m, \( n = 10 \) venules) and interconnecting capillaries (diameter = 8.7 \( \pm \) 0.6 \( \mu \)m, \( n = 10 \) capillaries; Fig. 3, A and B). The arterioles flowed from the nasal and temporal points of the iris margin to the pupillary center. The venules flowed from the pupillary center to the dorsal and ventral points of the iris margin (Fig. 3B). During the process of iris constriction, which was induced by applying 0.005% phystostigmine, the iris moved to the pupillary center, thereby bending the PM vessels at the iris margin, within which the blood flow was blocked over a large area of the PM (Fig. 3, C, D, F, G, and I; see also Supplemental Movie found at http://ajpregu.physiology.org/cgi/content/full/00602.2005/DC1). By contrast, during the subsequent iris dilation, which was induced by applying 0.5% tropicamide and 0.5% phenylephrine hydrochloride, the iris moved to the periphery, thus expanding the PM vessels, within which the blood flow was consequently restored (Fig. 3, E, H, and I; see also Supplemental Movie). The

![Fig. 3. Vascular conformation, blood-flow direction and the effects of iris movement on hemodynamics within the PM. A: diagrammatic illustrations of the anterior segment of the eye and a low-magnification view of the PM at postnatal (P) day 10 (P10). B: schematic showing the blood-flow direction (solid arrows) in the PM depicted in A. The red vessels indicate the direction of blood flow from the nasal and temporal points of the iris margin to the center of the pupil area. The blue vessels indicate the direction of blood flow from the center of the pupil to the dorsal and ventral points of the iris margin. A, arteriole; C, capillary; V, venule. C–E: low-magnification views of PMs at P12 under control conditions (C) or with a constricted (D) or dilated (E) iris. Scale bar: 1 mm. F–H: higher magnification images of C, D, and E, respectively: blue dotted lines indicate the iris margins; blue arrows show constrictive (D) and dilative (E) iris movements; red arrows show the same vessel under control conditions (F), blood-flow cessation (G) and resumption (H); and an asterisk (F) indicates the vessel that was evaluated for blood-flow velocity (I). Scale bar: 100 \( \mu \)m. I: Representative graph showing changes in the blood flow velocity during iris movement. Error bars indicate means \( \pm \) SE.

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velocity of blood flow in the PM ceased in conjunction with iris constriction, but 2 min after the instillation of the mydriatic drops, the velocity had recovered and exceeded 600 μm/s (Fig. 3f).

Continuous inhibition of iris movement suppressed apoptosis in the PM. Continuous inhibition of iris movement resulted in the persistence of the PM (Fig. 4A–C). At P12, the residual capillary density in the PM, in which iris movement had been inhibited, was significantly higher than control \((P < 0.0001\), 170.4 ± 5.5 and 69.8 ± 6.9 junctions, respectively; Fig. 4A). The results of the analysis of apoptotic cells in the PM with TUNEL were in good agreement with those described above: when iris movement was inhibited, vascular apoptosis was suppressed significantly \((P < 0.01\), the proportions of apoptotic cells were 4.4 ± 0.6% in the control group and 0.6 ± 0.2% after inhibition of iris movement; Fig. 4D). The distribution of apoptotic cells in the PM was diffuse and showed no apparent localization in the control group or the group in which iris movement had been inhibited (Fig. 4D). We confirmed that the instillation of not only atropine sulfate but also other mydriatics (e.g., parasympathomimetic agents, such as cyclopentolate hydrochloride and tropicamide, and sympathomimetic agents, such as phenylephrine hydrochloride) inhibited the regression of the PM.

Continuous inhibition of iris movement suppressed the migration of macrophages. To clarify the contribution of cellular factor, such as macrophages, to the regression of the PM, we investigated whether the continuous inhibition of iris movement affected the migration of macrophages toward the PM. The proportion of macrophages in the PM in which iris movement had been inhibited was significantly lower than that in the controls \((P < 0.05\), 9.0 ± 1.1% and 14.6 ± 0.9%, respectively; Fig. 4E).

Fig. 4. Effects of iris movement on regression of the PM, apoptosis, and the migration of macrophages. A: capillary density of the PM of Wistar rat neonates at P12. An asterisk indicates \(P < 0.0001\). B and C: low-magnification views of PMs of Wistar rat neonates from the control group at P12 (B) and after the inhibition of iris movement at P12 (C). Scale bars: 1 mm. D: double labeling of PMs for deoxynucleotidyl dUTP nick-end labeling (TUNEL; red) and 4,6 diamidino-2-phenyl-indole dihydrochloride (DAPI; blue) at P12. The graph shows the proportions of apoptotic cells at P12. Scale bar: 100 μm. \(*P < 0.01\). E: double labeling of PMs for ED-1 (red) and DAPI (blue). The graph shows the proportion of ED-1-positive cells at P12. Scale bar: 100 μm. \(*P < 0.05\). Error bars indicate means ± SE.
Continuous inhibition of iris movement suppressed PM regression in pigmented rats. Continuous inhibition of iris movement resulted in the persistence of the PM not only in albino rats (Wistar rat) but also in pigmented rats (Brown Norway rat) (Fig. 5, A–C). At P56, when the rats reached adulthood, the PMs of the atropine sulfate-treated rats persisted, the blood flow was maintained and the vascular endothelial cells (VECs) had a normal histological appearance (Fig. 5, C, F, and H). By contrast, the VECs in the control group showed a typical apoptotic morphology, which was characterized by a decrease in cell size and the condensation of the nucleus, even at P12 (Fig. 5G). The PMs of the atropine sulfate-treated rats remained partially viable for more than 50 days after the inhibition was relieved at P56 (that is, for more than 100 days after birth).

**DISCUSSION**

The continuous inhibition of iris movement by the instillation of mydriatic drops, which abolished this activity, provided strong evidence that iris movement induces regression of the PM. Here, we consider two possible mechanisms that might explain how iris movement induces vascular apoptosis in the PM. The first possibility is that the mechanical disruptions of cell-to-cell interactions, which are induced by iris movement, result in vascular apoptosis. A previous study showed that the disruption of VEC-pericyte associations resulted in excessive regression of the retinal vessels (2). The second possibility is that flow changes in the PM, such as cessation and resumption, in conjunction with iris movements, induce vascular apoptosis. According to our in vivo observations, the bending and expansion of the PM vessels together with iris movement occurred only near the iris margin (Fig. 3, C–H; see also Supplemental Movie). By contrast, movement of the iris induced the cessation and resumption of blood flow over a large area of the PM (Fig. 3, G and H; see also Supplemental Movie). The distribution of apoptotic cells in the regressing PM was diffuse and showed no apparent localization (e.g., near the iris margin; Fig. 4D). The cessation and resumption of blood flow are known to induce apoptosis in VECs via the production of reactive oxygen species and apoptotic ligands (3, 8, 14, 24). A reduction in blood flow (that is, a decrease in shear stress) has also been shown to trigger apoptosis in VECs (10). Thus we propose that iris movement induces the regression of the PM, not by causing mechanical damage to the vasculature, but by changing the blood flow within it. Ideally, to obtain further evidence that iris-mediated flow changes per se are responsible for the

![Fig. 5. Persistence of the PM after the continuous inhibition of iris movement in pigmented rats. (A–C) Low-magnification views of PMs of Brown Norway rat neonates from the control group at P14 (A) and after the inhibition of iris movement at P14 (B) and P56 (C). Most of the capillary meshwork of the PM has disappeared in A, but persists (white arrows) in B and C. D–F: rats from the groups shown in A, B, and C, respectively. Scale bar: 3 cm. G and H: electron-microscopic observation of apoptotic vascular endothelial cells (VECs) at P12 in the control group (G). Electron-microscopic observation of VECs at P56 after the inhibition of iris movement, showing normal morphology (H). L, capillary lumen; N, nucleus. Scale bars: 1 μm. I: diagrammatic illustrations that explain the mechanism of how the iris movement induces the blood flow cessation and resumption within the PM. Red arrowheads show the border where the PM and the lens surface meet, blue arrowheads show the iris margin, and black arrows show constrictive and dilative iris movements. Blood flow within the PM is shown in red.](http://ajpregu.physiology.org/)}
regression of the PM, we will need to confirm that flow changes with various causes under the continuous inhibition of iris movement can induce vascular apoptosis. However, in the current study, it was difficult to control blood flow without using surgically invasive procedures, because the eyes of rat neonates are extremely small and the PM is located in the anterior chamber, which is filled with aqueous humor. Further refinements of the methods employed here, including the use of larger animals, will therefore be necessary.

Our original high-resolution intravital video microscope revealed for the first time that constriction and dilation of the iris causes blood-flow cessation and resumption within the PM. Latker and Kuwabara (18) mentioned that the vascular network of PM adheres to the anterior surface of the lens by the end of the first postnatal week. To explain the mechanism for iris-produced blood flow changes in the PM, we believe the adhesion status of the PM to the anterior lens surface is important (Fig. 5I). On the basis of our in vivo observations, during the process of iris constriction, the iris moves to the pupillary center with bending of the PM vessels at iris margin. However, the iris cannot move beyond the edge of the area where the PM meets and adheres to the anterior surface of the lens; therefore, severe kinking occurs in the vasculature at this border, and the blood flow is blocked over a large area of the PM. By contrast, during the process of iris dilation, the iris moves to the periphery, thus expanding the PM vessels, within which the blood flow is consequently restored. One can argue that the direct vasoactive effects of eye drops might induce the blood flow changes, especially flow cessation along with iris constriction. However, Matsuo and Smelser (19) revealed that the PM is composed of vascular endothelial cells, thin basement membrane, pericytes, and a few collagen fibrils, but not of vascular smooth muscle cells. Our results from electron microscopic study support their findings. Because there is no vascular smooth muscle cell in the PM, it seems unlikely that the eye drops can cause vasoconstriction of the PM vessels. Indeed, during the process of iris constriction, we did not find vasoconstriction of the PM vessels, which is enough to cause blood flow cessation in the PM (Fig. 3, C, D, F, and G; see also Supplemental Movie). There is yet another possibility that ulcerated arteries, including the major arterial circle of iris, which are the trunks of PM vessels, might reduce the blood flow of the PM by constricting their smooth muscles (1, 25). However, these smooth muscles are constricted only by sympathetic stimulation, which induces iris dilation (1, 25). Therefore, the opposing vasoactive effect of parasympathetic stimulation, which induces iris constriction, should not constrict smooth muscles and decrease the blood flow in the PM.

Our findings have demonstrated that iris movement is the major factor initiating the first step of PM regression, while macrophages contribute to subsequent steps in the process. Lang and colleagues (5, 15, 16, 20, 21) propose that the initial apoptosis of VECs in the PM is induced by macrophages. However, little was previously known about the trigger that initiates macrophage migration toward the PM. In the current study, inhibiting iris movement suppressed the migration of macrophages (Fig. 4E) and resulted in the persistence of the PM. Previous studies using alternative experimental systems have indicated that the cessation and resumption of blood flow can induce the migration of macrophages (6, 17). Although further investigations are needed, our results were consistent with this theory, leading us to conclude that iris-mediated changes in blood flow activates macrophage migration. Using our persistent PM model, we are now trying to reveal the interaction between PM regression and other cellular and molecular factors—such as VEGF, bone morphogenetic protein, and reactive oxygen species, which are expected to participate in the process of developmental vascular apoptosis (12, 21).

In conclusion, we have revealed a causal relationship between iris movement and regression of the PM, and we have defined a previously unknown function of the iris during the developmental period. Although the mechanisms of the regression of other intraocular vasculature, such as the hyaloid artery and the tunica vasculosa lentis, are not fully understood, the present study demonstrates the importance of the physiological interactions between tissues—in this case, the iris and the PM—in developmental vascular apoptosis in the eye.

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REFERENCES