An anti-angiogenic state in mice and humans with retinal photoreceptor cell degeneration

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Abnormal angiogenesis accompanies many pathological conditions including cancer, inflammation, and eye diseases. Proliferative retinopathy because of retinal neovascularization is a leading cause of blindness in developed countries. Another major cause of irreversible vision loss is retinitis pigmentosa, a group of diseases characterized by progressive photoreceptor cell degeneration. Interestingly, anecdotal evidence has long suggested that proliferative diabetic retinopathy is rarely associated clinically with retinitis pigmentosa. Here we show that neonatal mice with classic inherited retinal degeneration (*Pdeb*^{rd1}/*Pdeb*^{rd1}) fail to mount reactive retinal neovascularization in a mouse model of oxygen-induced proliferative retinopathy. We also present a comparable human paradigm: spontaneous regression of retinal neovascularization associated with long-standing diabetes mellitus occurs when retinitis pigmentosa becomes clinically evident. Both mouse and human data indicate that reactive retinal neovascularization either fails to develop or regresses when the number of photoreceptor cells is markedly reduced. Our findings support the hypothesis that a functional mechanism underlying this anti-angiogenic state is failure of the predicted up-regulation of vascular endothelial growth factor, although other growth factors may also be involved. Preventive and therapeutic strategies against both proliferative and degenerative retinopathies may emerge from this work.

Excessive formation of new blood vessels in the retina is considered a hallmark of ischemic retinopathies such as diabetic retinopathy, a leading cause of blindness in the United States and Europe. Moreover, ocular neovascularization is considered a common etiological factor in diseases ranging widely in age of onset, from retinopathy of prematurity in oxygen-treated infants, to sickle cell disease and retinal venous occlusions seen in adults, to age-related macular degeneration observed in the elderly (1, 2).

The present investigation offers insight into the mechanism and possible prevention of retinal neovascularization. The study utilizes two well-established mouse models of disease. In the first, a mouse model of oxygen-induced ischemic retinopathy, mice are exposed to 75% oxygen (O_2) from postnatal day 7 (P7) to P12, after which time they are returned to room air. Their retinas are analyzed 5–9 days later (P17–P21), by which time neovascularization has supervened on the retinal surface (3). The exposure of neonatal mice to 75% O_2 causes vasoconstriction of the central retinal blood vessels. The decreased retinal perfusion, along with the return of the mice to room air, is believed to lead to a relative retinal tissue hypoxia and ischemia, resulting in marked retinal neovascularization (3–5).

The mechanism underlying neovascularization in this animal model and in certain human diseases is thought to involve, among other factors, a hypoxia-driven up-regulation of vascular endothelial growth factor (VEGF; refs. 4–10). Overexpression of VEGF in the retina is sufficient to cause intraretinal and subretinal neovascularization (8), whereas inhibition of VEGF expression or activity inhibits retinal neovascularization (9). VEGF, a 45-kDa glycoprotein that binds to several transmembrane tyrosine kinase receptors, is produced by glial cells of the neural retina, such as specialized astrocytes, including Muller cells, among other cell types (4–6). VEGF expression in the retina decreases within 6 h of exposure to 75% oxygen and remains decreased for the duration of the hyperoxia. In contrast, an increase in retinal VEGF expression is observed between 6 and 12 h after the return to room air, and such expression remains elevated during development of the neovascularization. Therefore, VEGF levels appear to play a dual role in this retinopathy model: a down-regulation of VEGF by hyperoxia induces blood vessel regression, whereas subsequent up-regulation of VEGF leads to retinal neovascularization (5, 10).

The second mouse model used in this study is the classic autosomal recessive inherited degenerative disease of photoreceptor cells known as retinal degeneration, Pdeb^{rd1}. This disease is caused by a nonsense mutation in the β subunit of the rod photoreceptor cell-specific phosphodiesterase (11–14). Light absorption by rhodopsin activates transducin, a G protein, which in turn promotes cGMP hydrolysis by the specific phosphodiesterase, leading to hyperpolarization of rod photoreceptor cells (15). The widely distributed *Pdeb*^{rd1} mutation (11) can be traced back directly to Keeler's rodless mutation (16), as shown by analysis of DNA extracted from Keeler's original microscope slides 70 years later (17). The retinal development in *Pdeb*^{rd1}/*Pdeb*^{rd1} mice proceeds normally until P11. At that time, the development of photoreceptor cell outer segments arrests, and the rod cell nuclei, inner segments, and outer segments begin to degenerate. Photoreceptor cell degeneration then proceeds rapidly, and exceeds 80% by P15, and 90% by P21 (ref. 18). By P25–P30 only one sparsely populated row of photoreceptor cell nuclei remains, and the outer segments have disappeared. By the beginning of the fourth postnatal week, most surviving photoreceptor cells are cone cells (19, 20). Apoptosis of the photoreceptor cell is the final pathogenic event common to all animal models of retinal degeneration (21, 22). In addition to the primary photoreceptor cell loss, *Pdeb*^{rd1} mutant mice (23) and patients with retinitis pigmentosa (24) may also have an altered retinal blood flow.

Materials and Methods

Animals. This study adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in

Abbreviations: P*n*, postnatal day *n*; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor; wt, wild type; H&E, hematoxylin and eosin.

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Ophthalmic and Vision Research. Mouse experiments were approved by the Animal Care and Use Committees of the Burnham Institute and of the University of Texas M. D. Anderson Cancer Center. The strain of inbred congenic C57BL/6 mice carrying the *Pdeb*^{rd1} mutation has been described (25). We used mice at the F77N2F14–16 generations. C57BL/6 + /+ wt mice (Harlan, Indianapolis) were used as controls.

Induction of Retinal Neovascularization. P7 mouse pups with their nursing mothers were exposed to 75% oxygen for 5 days. Mice were returned to room air (20.8% O_2) on P12. For histological analysis, mice were killed between P17 and P21 and eyes were enucleated and fixed in 4% paraformaldehyde in PBS overnight at 4°C. For RNA isolation, mice were killed and their eyes were enucleated on P12 either immediately or 12 h after return to room air from 75% O_2. Retinas were dissected and stored in TRI reagent (Sigma) at −80°C.

Histological and Immunohistochemical Analysis. Fixed and alcohol dehydrated eyes were embedded in paraffin and serially sectioned at 5 μm. Tissue sections were stained either with hematoxylin and eosin (H&E) or immunostained with an anti-von Willebrand factor antibody (Dako) according to the manufacturer's instructions. Endothelial cell nuclei on the vitreous side of the internal limiting membrane were counted (3). At least six H&E-stained sections were evaluated per eye, and the average number of nuclei was counted from at least eight eyes for each experimental condition. Student's *t* test was used to determine whether the differences observed were statistically significant. All experiments were repeated at least three times under similar conditions.

Northern Blot Analysis and *in Situ* Hybridization. RNA was isolated from mouse retinas by using the TRI reagent according to the manufacturer's instructions. Total retinal tissue RNA (8 μg per sample) from each time point was electrophoresed on a 1% agarose gel containing 6% formaldehyde (26). RNA was transferred to nylon membranes and hybridized with a ^{32}P-labeled $VEGF_{165}$ cDNA probe (26). Densitometry data were acquired and analyzed by using a FluorChem imager and software (Alpha Innotech, San Leandro, CA). Colorimetric *in situ* hybridization of paraffin-embedded eyes was performed with hyperbiotinylated oligoprobes (27).

Results

Abolishment of Reactive Retinal Neovascularization in Young Mice with Inherited Retinal Degeneration. To test the angiogenic response of the Pdeb^{rd1} mutant retinas in response to ischemia, we designed experiments with the mouse models of O_2-induced retinopathy and retinal degeneration simultaneously. Combination of the models produced the surprising finding that the reactive retinal neovascularization characteristic of normal young mice exposed to high O_2 levels, and observed in wild-type (wt) and heterozygous animals, failed to occur in *Pdeb*^{rd1} homozygotes. Neovascularization was quantified by counting vascular endothelial cell nuclei protruding into the vitreous space from at least six sections of 8–36 eyes in five independent experiments (Table 1). Extensive induction of retinal neovascularization (40.0 ± 3.2 endothelial cell nuclei per eye section) was seen in C57BL/6 +/+ wt mice on P17 after 75% oxygen treatment from P7 to P12 (Fig. 1*b*) and in heterozygous +/*Pdeb*^{rd1} mice (data not shown). Virtually no endothelial cell nuclei (0.4 ± 0.1 endothelial cell nuclei per eye section) were seen in the *Pdeb*^{rd1}/*Pdeb*^{rd1} retinas on P17 after exposure to 75% oxygen from P7 to P12 (Fig. 1*d*). At this time only a few layers of nuclei remained in the photoreceptor cell layer. Also, no endothelial cell nuclei were seen on or after P21, ruling out the possibility of delayed retinal neovascularization (data not shown). No endothelial cell nuclei were seen on P17 in either wt or *Pdeb*^{rd1}/*Pdeb*^{rd1} mice exposed only to room air (Fig. 1 *a* and *c*). Staining for von Willebrand factor confirmed that the cells protruding into the vitreous space of wt mice treated with 75% oxygen were indeed endothelial cells (Fig. 1 *e* and *g*) and that such cells were almost completely confined to the neural retina in *Pdeb*^{rd1}/*Pdeb*^{rd1} homozygotes (Fig. 1 *g* and *h*).

Table. 1. Effect of O_2-induced retinal neovascularization in wt and *Pdeb*^{rd1}/*Pdeb*^{rd1} mice

	Eyes with retinal neovascularization/ total number of eyes examined	
Mice	+/+	*Pdeb*^{rd1}/*Pdeb*^{rd1}
Control (room air, P1–P17)	0/8	0/12
Experimental (75% O_2, P7–P12)	36/36	0/34*

Results were compiled from five independent experiments. Retinas were examined on P17. *, $P < 0.0001$.

Failure of the Predicted Up-Regulation of VEGF in Mice with Inherited Retinal Degeneration. VEGF has been suggested to be one of the key angiogenic factors in oxygen-induced retinal neovascularization (4–10). We hypothesized that differences in VEGF expression could play a role in the lack of neovascularization in the retinas of *Pdeb*^{rd1}/*Pdeb*^{rd1} mice and examined VEGF expression in retinal tissue by Northern blot analysis (Fig. 2). Total RNAs from wt and *Pdeb*^{rd1}/*Pdeb*^{rd1} mouse retinas were analyzed on P12 after exposing mice for 5 days to either 75% O_2 or room air. A decline in VEGF expression was seen during exposure to hyperoxia. This decrease was followed by a 150% increase in the VEGF expression in wt mouse retinas observed 12 h after the return to room air after 75% O_2 exposure, compared with that seen after exposure to room air only. In *Pdeb*^{rd1}/*Pdeb*^{rd1} mice, retinal VEGF expression remained low and unchanged even after exposure to 75% O_2 for 5 days, comparable to retinas of similar (otherwise isogenic) mice exposed only to room air. To determine whether inhibition of neovascularization was a consequence of an altered spatial expression pattern of VEGF rather than an overall alteration in VEGF expression levels in the *Pdeb*^{rd1}/*Pdeb*^{rd1} mouse retina, we analyzed VEGF expression in the retina by *in situ* hybridization. Tissue sections from wt and *Pdeb*^{rd1}/*Pdeb*^{rd1} mouse eyes were evaluated on P12, 12 h after of exposure to either 75% O_2 or room air for 5 days. Slightly higher VEGF mRNA levels were seen in the inner nuclear layer and in the inner plexiform layer of wt mouse retinas on P12, after 12 h in room air after 75% O_2 exposure. These expression patterns are consistent with previous studies (4), but a comparable increase in VEGF expression was not seen in any region in *Pdeb*^{rd1}/*Pdeb*^{rd1} mouse retinas after 75% O_2 exposure (data not shown).

Regression of Diabetic Retinopathy in Some Patients with Retinitis Pigmentosa. Are there clinical counterparts to this mouse experiment in which an exogenous stimulus of pathological formation of new retinal blood vessels fails in the presence of advanced photoreceptor cell degeneration? Here we present a clinical case in which proliferative retinopathy regressed spontaneously in a diabetic patient with concurrent retinitis pigmentosa. On fundus examination of a 36-year-old woman, diagnosed with type I diabetes mellitus for the past 34 years, we observed, in both eyes, inactive fibroglial membranes projecting into the vitreous space from the optic discs (Fig. 3). This pattern was consistent with regressed retinal neovascularization, often observed in cases of patients with proliferative diabetic retinopathy after successful laser treatment (15). However, this patient had never received laser treatment. In the periphery and midperiphery of the

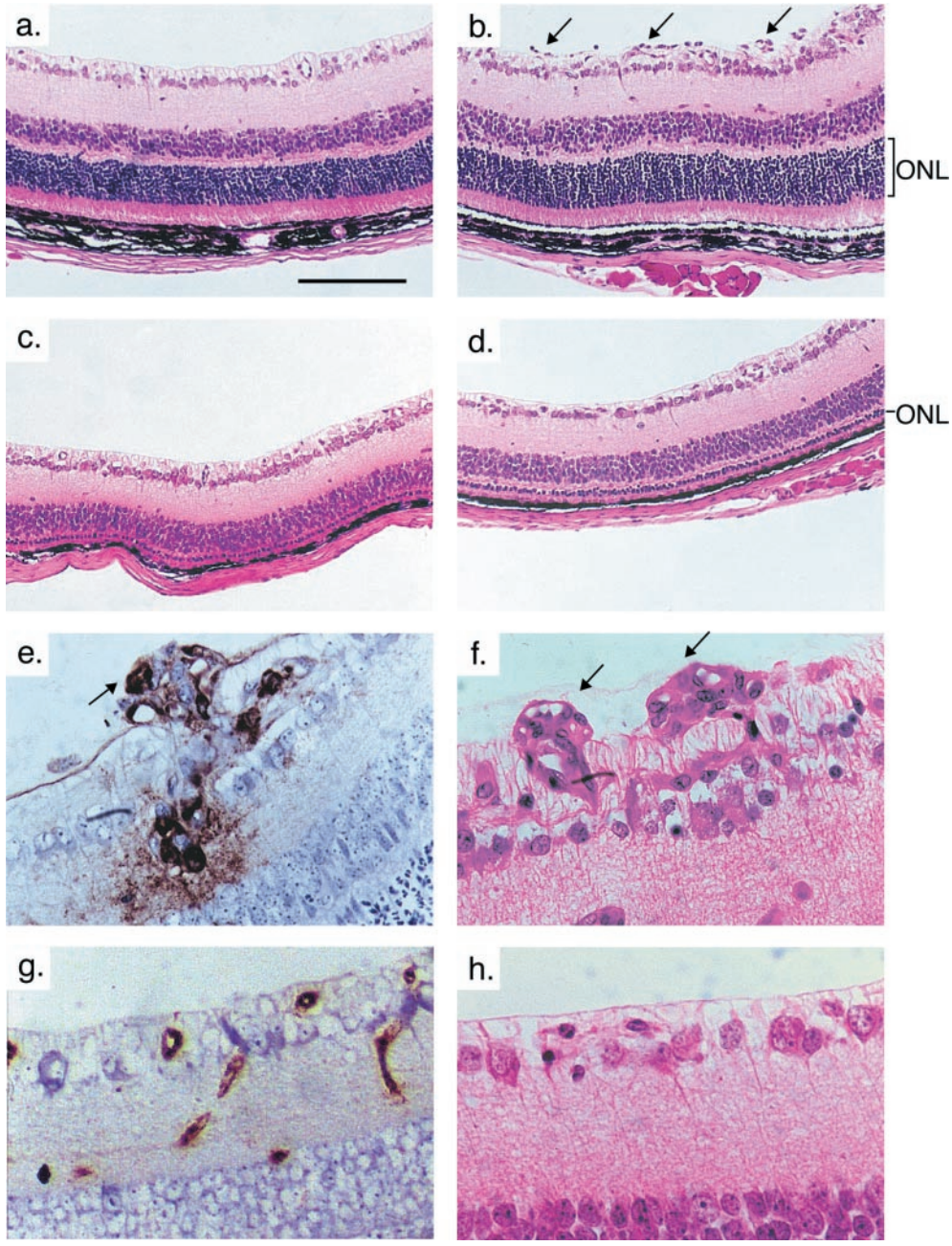

Fig. 1. Effect of relative hypoxia on C57BL/6 +/+ wt and on *Pdeb*^{rd1}/*Pdeb*^{rd1} mutant mouse retinas. (*a*) Wild-type retina on P17 of a mouse kept continuously in room air. (*b*) Wt retina on P17 after exposure to 75% oxygen for 5 days from P7 to P12. A large number of new blood vessels are seen protruding into the vitreous space. (*c*) *Pdeb*^{rd1}/*Pdeb*^{rd1} retina on P17 when mouse has been kept in room air. (*d*) *Pdeb*^{rd1}/*Pdeb*^{rd1} retina on P17 after exposure to 75% oxygen for 5 days from P7 to P12. No new blood vessels are seen protruding the vitreous space. (*e*) Anti-von Willebrand factor (vWF) antibody-immunostained section of wt retina on P17 after exposure to 75% oxygen from P7 to P12. (*f*) Detail of an H&E-stained section of a typical new blood vessel in a wt retina after exposure to 75% oxygen from P7 to P12. (*g*) vWF-antibody-stained section of *Pdeb*^{rd1}/*Pdeb*^{rd1} retina on P17 after exposure to 75% oxygen from P7 to P12. (*h*) Detail of an H&E-stained section of a *Pdeb*^{rd1}/*Pdeb*^{rd1} retina on P17 after exposure to 75% oxygen from P7 to P12. Arrows point to endothelial cell nuclei. (Scale bar: *a*–*d*, 100 μm; *e*–*h*, 35 μm.) ONL, outer nuclear layer.

fundus, attenuated vasculature and atrophic retina with granular and bone spicule pigmentary changes were observed, consistent with a diagnosis of retinitis pigmentosa (Fig. 3) and confirmed by a virtually flat electroretinogram (i.e., less than 10 μV; data not shown). In nondiabetics with retinitis pigmentosa, spontaneous regression of optic disk neovascularization caused by an unknown mechanism can also occur (28). This example and other sporadic clinical case reports (28–30) suggest that, in the clinical setting of rod photoreceptor cell degeneration, proliferative retinopathies may fail to develop or regress early.

Discussion

The pathogenesis of neovascularization in ischemic retinopathies is best considered in the context of vasculogenesis in the normal developing retina. Blood vessels enter the back of the embryonic eye at the eye-cup stage and reach the vitreal surface via the choroidal fissure. This fissure closes around the developing optic nerve and the blood vessels close to the vitreal surface, which supply the innermost part of the central retina. As the retina expands during and after the fetal period, the vessels branch and grow radially outward toward the retinal periphery. Astrocytes

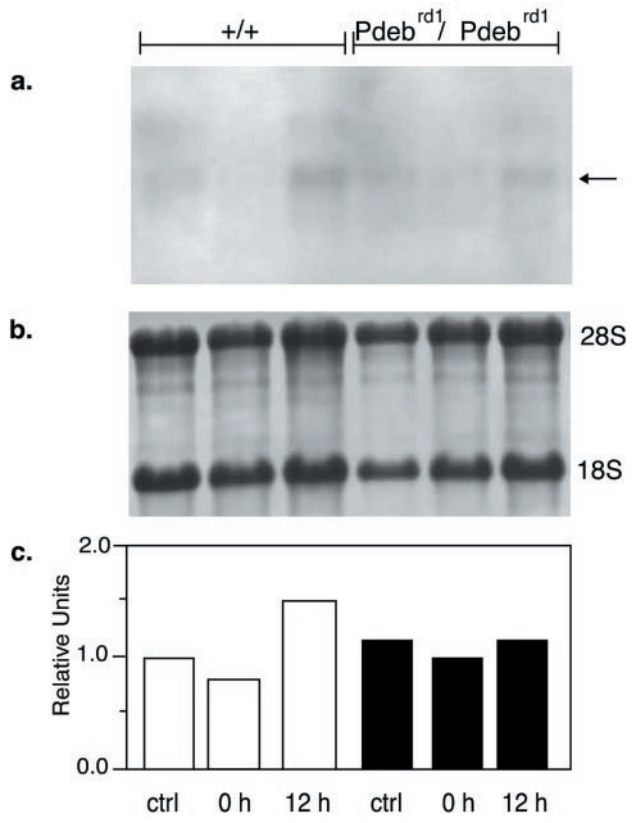

Fig. 2. VEGF expression in wt mouse and *Pdeb*^{rd1}/*Pdeb*^{rd1} mouse retinas. (*a*) Northern blot analysis of VEGF expression in wt (lanes 1–3) and *Pdeb*^{rd1}/*Pdeb*^{rd1} (lanes 4–6) mouse retina. Wild-type and *Pdeb*^{rd1}/*Pdeb*^{rd1} mice were exposed to 75% O_2 from P7 to P12. On P12, retinal RNA was isolated immediately (0 h; lanes 2 and 5) or 12 h (lanes 3 and 6) after return to room air from 75% O_2. Retinal VEGF expression was quantified also from retinas of mice kept only in room air until P12 (control; lanes 1 and 4). Arrow indicates VEGF transcript (3800 bp). (*b*) 28S and 18S ribosomal markers serve as loading controls. (*c*) Integrated density values of VEGF transcripts shown in *a* and *b* were quantified. The baseline value for VEGF expression in wt mice kept only in room air until P12 was set to 1.0. Standard deviations were typically less than 10% of the mean. A representative experiment is shown.

lie in the avascular zone just ahead of the radially spreading vessels, and are thought to stimulate and control the direction of vessel growth by local release of VEGF (4–6, 15, 31). Astrocytes also grow inward into the inner plexiform and inner nuclear layers of the developing retina, and they stimulate the growth of immature vessels inward in their path. Once blood vessels mature to the stage, at which point they are invested with peri-endothelial cells ("pericytes"), they lose responsiveness to VEGF (32). Another set of blood vessels supplies the choroid, which is just external to the pigment epithelial layer. The pigment epithelial layer itself and the entire length of the photoreceptor cells, from their synaptic endings in the outer plexiform layer, through their nuclei in the outer nuclear layer, to their specialized inner and outer segments close to the pigment epithelium, are normally avascular. In experimental or clinical contexts in which retinal hypoxia induces VEGF expression, new blood vessels will form either on the inner neural retina in young subjects or, in some older subjects, from the choroid across the pigment epithelium (31). In contrast, when VEGF basal expression drops, endothelial cells undergo apoptosis (5) and retinal vasculature regresses, resulting in a reduced retinal blood supply.

Recently, Arden proposed (33) the intriguing hypothesis that the high oxygen consumption of dark-adapted rod cells is the driving force of inner retinal hypoxia, with subsequent VEGF production leading to retinal neovascularization in ischemic retinopathies. This hypothesis was indirectly supported by the observation that diabetic retinopathy rarely occurs in retinitis pigmentosa patients (28–30, 33, 34) and by the clinical success of panretinal photocoagulation, a treatment that destroys a large number of rod photoreceptor cells and reduces intraocular VEGF levels (35). Our findings show that degeneration of rod cells leads to a total lack of reactive retinal neovascularization, accompanied by a failure in the expected VEGF up-regulation. Taken together, these observations of *Pdeb*^{rd1}/*Pdeb*^{rd1} mice and a human patient afflicted with both diabetes mellitus and retinitis pigmentosa provide direct experimental and mechanistic evidence in support of Arden's hypothesis (33) and suggest that VEGF is a primary link between rod cell numbers and retinal neovascularization. Indeed, it is tempting to speculate that reducing the metabolic rate of rod cells at critical time windows may improve the incidence of retinopathy of prematurity or perhaps slow the progression of diabetic retinopathy in

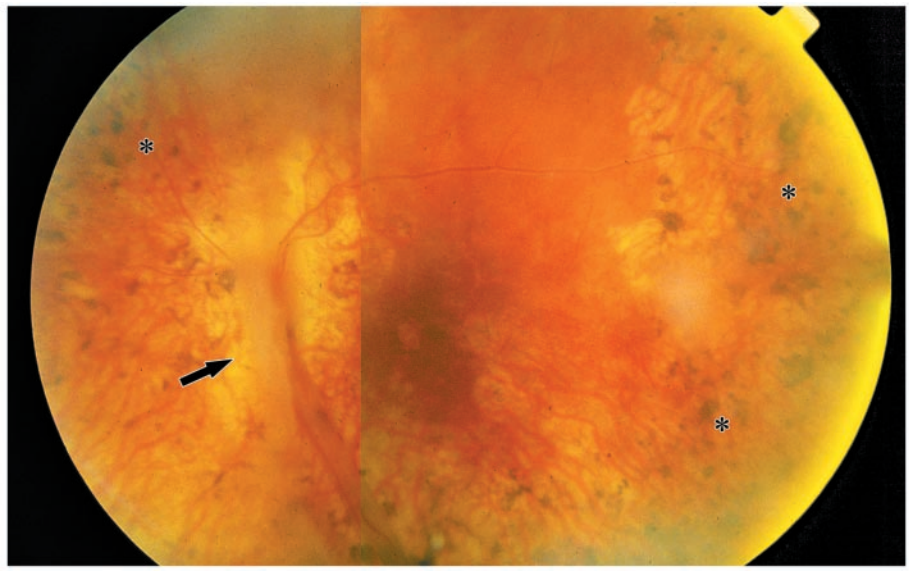

Fig. 3. Spontaneously regressed optic disk neovascularization (arrow) in a 36-year-old woman with concurrent type I diabetes mellitus and retinitis pigmentosa. Note the granular and "bone spicule-like" pigmentary changes in the retina (asterisks) consistent with a diagnosis of retinitis pigmentosa.

adults. For instance, it is conceivable that increased exposure of premature neonates to light may reduce O_2 consumption by rod photoreceptor cells and retinal hypoxia, ultimately improving their retinopathy. Paradoxically, such reasoning challenges the current recommendation to decrease ambient light exposure in that setting, which has actually failed to prevent retinopathy of prematurity (36).

These observations notwithstanding, VEGF is not the only angiogenic mediator whose production is affected by changes in O_2 tension (37–42). Moreover, VEGF inhibitors and blockers can only partially halt angiogenesis in the retinopathy of prematurity model (9), and not all of patients with diabetic retinopathy show a rise in VEGF (35). Thus, the total absence of retinal neovascularization in homozygous *Pdeb*^{rd1} mice argues that the degeneration of photoreceptor cells may have further effects on angiogenesis that are not VEGF-mediated. Our data do not rule out a possible role for other angiogenic factors known to be regulated by hypoxia (37–40), such as transforming growth factor-β, insulin-like growth factor-1, placental growth factor, and interleukin-8, and more studies are needed to clarify these multiple interactions in the retina.

Finally, this work highlights the recent awareness that growth factors and inhibitors may be involved in coordinating neural and vascular components of the retina by functioning simultaneously as photoreceptor cell survival factors and endothelial cell regulators. Basic fibroblast growth factor (bFGF) is elevated in *Pdeb*^{rd1}/*Pdeb*^{rd1} mice several days before photoreceptor cell death (43), and intravitreous injection of bFGF delays the onset of photoreceptor cell degeneration in selected animal models (44). Pigment epithelium-derived factor (PEDF), which is encoded by a gene that is closely linked to the *Pdeb* locus (45), is a survival factor for photoreceptor cells (46) and has been proposed also to play an anti-angiogenic role in the retina (41). Given that PEDF concentration is highest in the matrix surrounding the photoreceptor cell layer (41, 47), which undergoes apoptosis in *Pdeb*^{rd1}/*Pdeb*^{rd1} mice (21, 22), one might expect that a loss of PEDF would be correlated with an increase rather than a decrease in retinal angiogenesis. In our system, preliminary data based on immunostaining failed to show a correlation between PEDF and neovascularization (data not shown) and suggest that PEDF does not play a major role in the phenomenon described here. However, it is possible that PEDF or other angiogenesis inhibitors are released during the photoreceptor cell apoptotic process, which may contribute to the lack of retinal neovascularization. Roles for additional factors are suggested by the dramatic but unexplained variations in timing of retinal capillary growth into the pigment epithelial cell layer among mutant mice, which share a similarity in timing of photoreceptor cell degeneration (48). Finally, the neurotransmitter dopamine has been recently shown to inhibit VEGF-induced angiogenesis (49). However, dopamine synthesis and utilization are known to be suppressed at least in some mouse models of retinal degeneration (50).

In summary, we show that ischemia-induced neovascularization of the retina is abolished in a mouse strain with inherited photoreceptor cell degeneration. We also document that regression of established reactive retinal neovascularization caused by diabetes mellitus can occur in a subset of adult patients also afflicted with retinitis pigmentosa. This striking, previously unreported failure to mount a reactive retinal neovascularization response to potent exogenous stimuli is associated with an absence of the expected VEGF up-regulation in the retina. Our findings support the hypothesis that O_2 consumption by rod cells is a major driving force in ischemic retinal neovascularization and controls VEGF production. Additional trophic agents and cytokines are likely also to be involved in this complex biological phenomenon. Further characterization of this anti-angiogenic state in the retina may lead to therapeutic approaches against important eye diseases such as ischemic retinopathies and late complications of retinitis pigmentosa.

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