Ambient Hypoxia Reverses Retinal Vascular Attenuation in a Transgenic Mouse Model of Autosomal Dominant Retinitis Pigmentosa

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PURPOSE. Loss of retinal capillaries is an inherent component of late stage autosomal dominant retinitis pigmentosa (ADRP). This study examined the hypothetical role of tissue hypoxia in this vascular attenuation process and tested the potential of ambient hypoxia to reverse it.

METHODS. Transgenic mice expressing a mutant opsin gene with a 3-bp deletion of isoleucine at codon 255/256 were used. This model is characterized by early onset of a rapidly progressing retinal degeneration that by postnatal day (P)20 results in the loss of all but one row of photoreceptor nuclei. At P20 some mice were placed in 12% oxygen until they were euthanatized at P26. The remainder were maintained in normoxia and killed at the same age. Retinas were dissected, stained for ADPase, and flat-mounted.

RESULTS. Deep plexus capillary density was significantly different in normoxic normals versus transgenics at 20 days of age \((P \leq 0.005)\). An additional 65% reduction of capillary density occurred within the deep plexus of normoxic transgenics between P20 and P26 \((P \leq 0.005)\). Ambient hypoxia between days P20 and P26 reversed this trend, causing an increase in deep capillary plexus density of nearly 100% \((P \leq 0.001)\).

CONCLUSIONS. This model of ADRP demonstrates two important features of human retinitis pigmentosa: photoreceptor cell death and subsequent retinal capillary atrophy. Low ambient oxygen was used to reverse the capillary atrophy and to stimulate new capillary growth, implying that retinal oxygen tension may link these two features of the pathology. The implications of this study hold importance for strategies designed to treat retinitis pigmentosa with retinal cell transplantation. (Invest Ophthalmol Vis Sci. 2000;41:4007–4013)

One of the first and predominant opsin mutations correlated with retinitis pigmentosa (RP) was observed in Europe and reported in 1991 by Inglehearn and colleagues.1,2 The site of this mutation, which eliminates one of two sequential isoleucine residues at position 255 or 256, occurs within transmembrane helix 6 of the molecule.3 The effect of this autosomal dominant mutation is early childhood onset of night blindness, visual field restriction by 20 years of age, and daytime visual difficulty by the third decade of life.2 Fundus findings include areas of hypo- and hyperpigmentation, sparse pigment migration into the neuroretina, and progressive choroidal atrophy.

The classic appearance of moderately advanced RP also includes retinal vascular attenuation.4 This feature is prominently displayed in the transgenic mouse with a deletion of isoleucine at codon 255/256. Retinal vascular attenuation has been studied in three rodent strains with autosomal recessive gene defects leading to retinal degeneration, but to our knowledge this is the first study of vascular attenuation in a transgenic mouse model of autosomal dominant retinitis pigmentosa (ADRP). On the basis of the assumption that retinal hypoxia resulting from photoreceptor death contributes to retinal vessel atrophy in RP, we tested the potential of ambient hypoxia to reverse it in this mouse model.

METHODS

The Transgene

The I-255/256 mutation has been expressed in transgenic mice by PCR site-directed mutagenesis in exon 4 of the mouse opsin gene.6 In addition to the coding and intervening sequences, the transgene contained 6.0 kb of the promoter and 3.5 kb of the 3’ untranslated regions. The transgene is passed in Mendelian fashion, and heterozygotes with the mutant phenotype show rapid progression of rod photoreceptor cell death that leads to complete absence of a dark-adapted ERG b-wave by P20.6
Treatment Protocol

Four males, homozygous for the deletion, were bred with eight C57BL/6 females to produce heterozygotes with the mutant phenotype. Controls were matched for female breeder genotype and age. Using intraperitoneal overdose (100 mg/kg) of sodium pentobarbital, mice were killed at P15, P20, and P26 for comparison of the vasculature of transgenic and control retinas. Sample sizes for transgenic animals were 7, 7, and 6 for the three ages, respectively. Sample sizes for controls were 6, 6, and 6, respectively. In addition, groups of 15 normal and 17 transgenic mice were raised through 20 days of age, at which time 3 from each group were killed for retinal vascular assessment. Of the remainder, 8 normals and 14 transgenics were placed in a breathing atmosphere of 12% oxygen. These mice were killed (100 mg/kg sodium pentobarbital) at 26 days of age, along with the remaining 4 normoxic normal mice, and all were prepared for retinal vascular assessment. Experiments involving animals were approved by the Vanderbilt University School of Medicine Animal Care and Use Committee, and they adhered to the principles expressed in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Arrows in Figure 1 denote times mice were killed for the various experimental groups.

Retinas were dissected and stained for adenosine diphosphatase (ADPase) activity, and were viewed and photographed in flat mount as previously described for canine7 and rat retina.8 Briefly, ADPase histochemistry consists of incubation of dissected retinas at 37°C with lead nitrate, magnesium chloride, and adenosine diphosphate, followed by washing and staining with ammonium sulfide. This process causes ADPase activity to be associated with lead sulfide precipitate, which is limited primarily to vascular endothelium.9

Vascular Assessment

The density of deep and superficial capillary beds was quantified in digitized images of ADPase-stained retinas. A 0.4-mm² rectangular area of the midperipheral retina was converted to binary using Enhance 3.0 software (Microfrontier, Des Moines, IA). In cases where manual capillary tracing was necessary to accurately recreate the complete vessel network, the process was performed in a masked fashion. The ratio of black to white pixels in the 0.4-mm² area represents the capillary density in that retinal region. The calculations were made in each of the four retinal quadrants of all mice at 100× magnification where the superficial and deep capillaries could be discriminated easily. In Figure 2, the technique is shown at lower magnification to illustrate the location within each quadrant where measurements were made. The four quadrant values were averaged for each retina, and then the two retinal values were averaged for each mouse; finally, mean values from each mouse were averaged for each treatment group. In four retinas of three transgenic mice, the focal plane of the deep capillary network contained melanin pigment at one or more points of interest (the lower left panel of Fig. 5 illustrates the most extreme of these four cases). The affected quadrants in these four retinas (6 of 148 total retinal quadrants from transgenic mice) were eliminated from the analysis. Superficial net and deep net mean vessel densities were tested for distribution normality. Comparisons between treatment groups were made by analysis of variance with Scheffe’s post hoc procedure.

Additional mice from each treatment group were killed, and their retinas were processed for conventional histology. Briefly, the tissue was fixed in glutaraldehyde and osmium tetroxide, hydrated in graded ethanol, embedded in methacrylate, sectioned at 0.5 μm in transverse orientation, and stained with 0.5% toluidine blue.

RESULTS

Retinal Degeneration

Histologic comparison of the I-255/256 transgenic mice to normal mice showed a rapid and profound loss of photorecep-
tors (Fig. 3). The retinal photoreceptor complement of the transgenic mice was represented by one row of nuclei at P20, a time at which they produced no significant rod response. A very small cone b-wave persisted through P30.6

The mouse retina, like that of the human, contains a bilayered vasculature. Major veins and arteries reside just beneath the inner limiting membrane, as do secondary and tertiary arterioles (called the superficial vessel net). Venules and capillaries reside within the inner nuclear layer (called the deep net). Vasculogenesis was virtually complete at 15 days of age in the normal mouse (Fig. 4, left). Both the superficial and deep capillary networks extended to the retinal periphery. There was little or no pigment adherence upon retinal dissection. In the transgenic mouse at this age, some retinal arteries stopped short of the retinal periphery (Fig. 4, right). The deep capillary plexus, although it was already attenuated pan-retinally, was dense relative to its appearance 6 days later. Pigment adhered to the retina upon dissection, particularly in the periphery.

In the 20-day-old normal mouse, the retinal vasculature had a nearly adult appearance. Both deep and superficial capillary networks were fully arborized and the periarterial capillary-free zone was of adult proportions (Fig. 5, top). There was no pigment adherence upon retinal dissection. Transgenic mice at this age displayed a range of severity of retinal vasculopathy, from mild (Fig. 5, middle), with little pigment migration into the retina and marginal loss of deep capillaries, to severe (Fig. 5, bottom), with extensive penetration of the neural retina by pigment and little remaining of the deep capillary plexus. It is unknown if this spectrum of pathology represents a differential expression of the mutant opsin in heterozygotes. All transgenic mice exhibited some degree of perivascular pigmentary cuffing of deep capillaries at this age. There was also significant arterial constriction in transgenics at this age.

By 26 days of age, little of the deep capillary plexus remained in transgenic mice, and virtually every short expanse of deep capillaries was associated with some degree of pigment cuffing. The retina in Figure 6 (left) was photographed upside-down (sclerad surface up) to illustrate better the attenuation of deep vessels. At higher magnification (right), the melanin pigment surrounding the short capillary loops of the deep plexus could be observed readily.

**Treatment Comparison**

Figure 7 illustrates the vessel densities in retinas from each treatment group. Digitized images were pseudocolored, with superficial vessels (major arteries, major veins, arterioles, and...
arterial capillaries) in pink and deep vessels (venules and venous capillaries) in light blue. Transgenic retinas at 20 days of age showed attenuation of the deep vascular bed relative to the age-matched normal, whereas the superficial vascular bed remained relatively unchanged. At 26 days of age, transgenic retinas showed continued attenuation of deep capillaries and a slight (not significant) increase in superficial net density. Normal retinas at 20 days of age had a superficial vessel net comparable to that of transgenic retinas at 20 and 26 days of age, but the deep net was much more elaborate. Transgenic mice exposed to hypoxia (12% oxygen) from days 20 to 26 showed an increase in the densities of both the deep and superficial vascular beds during the hypoxic period. The new superficial plexus exhibited a gross architecture (branch angles and frequencies) typical of normal deep vessels.

Vascular Density

By 20 days of age, vessel density was significantly different in the deep plexus of normals versus transgenics ($P \leq 0.005$, Fig. 8). An additional 65% loss of deep plexus density occurred over the next 6 days in transgenic mice in room air ($P \leq 0.01$). However, when these 6 days were spent in ambient hypoxia, the deep plexus density increased to a level significantly different from age-matched normoxic transgenics ($P \leq 0.005$) and from littermate transgenics killed at 20 days (i.e., before hypoxia; $P \leq 0.01$). Superficial plexus density was not different between normals at 20 days of age and transgenics at 20 and 26 days of age, but it was significantly increased in the transgenics exposed to hypoxia ($P \leq 0.01$). No differences in vessel density were seen between normoxic normal mice at 20 versus 26 days of age, nor were densities different between these two groups and normals exposed to hypoxia between 20 and 26 days of age.
DISCUSSION

Because the I-255/256 mutation deletes an amino acid rather than substituting one, without changing the reading frame, it has the effect of shortening the sixth transmembrane domain. This may affect secondary structure and function of the gene product far beyond any significance that the deleted amino acid would have had alone. The single amino acid deletion has the effect of replacing each of the following 20 amino acids with the adjacent one. It would not be surprising to find that this protein is unable to form a stable 7-helical bundle, to bind the chromophore, or to proceed routinely from the endoplasmic reticulum. This would explain the rapid loss of photoreceptors that is observed in the mouse model and is presumed

![Image](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933216/)
FIGURE 8. Using the method illustrated in Figure 2, vessel densities were measured and are compared for each experimental group. Data are described as means; error bars, SDs. Confidence intervals were determined by analysis of variance and Scheffe’s post hoc test. *N20 vs. T20 deep plexus density; P ≤ 0.005. †T20 vs. T26 deep plexus density; P ≤ 0.01. ‡T26 vs. T26 with hypoxia deep plexus density; P ≤ 0.005. §T20 with hypoxia versus all other superficial vessel density; P ≤ 0.01.

to occur (as measured by ERG) in humans with the same mutation. Notably, the opsin protein in our mutant mouse has not been completely characterized to date. At this stage, we depend on the clear phenotype to support its suitability for studies of this type. It is more important in the present context to know that the vast majority of photoreceptors are lost in the model retina than it is to know the specific mechanism of that loss.

Pan-retinal laser photocoagulation is a proven treatment strategy that suppresses retinal neovascularization in diabetic retinopathy. The theory that the laser exerts its effect by increasing inner retinal oxygen is well supported by animal experiments using oxygen microelectrodes, which consistently demonstrated higher oxygen tensions over laser-treated retinal regions compared with untreated regions. This effect was evident in normoxic rabbits10 and miniature pigs11 and in hyperoxic monkeys12 and cats.13 Stefansson and colleagues14 extended these findings to humans using a fiberoptic oxygen probe in patients who underwent vitrectomies secondary to proliferative diabetic retinopathy. Vascular endothelial growth factor, an angiogenic cytokine induced by hypoxia and suspected to play a role in diabetes-induced and other forms of retinal neovascularization, was shown to be downregulated in laser- and cryoprobe-treated retinal areas.15,16 These studies differ from ours in that, in all cases, the neural elements of the subjects’ retinas were largely intact at the time retinal oxygen tensions were measured. Perhaps more pertinent, then, is a recent report describing experiments conducted in Abyssinian cats with an inherited retinal degeneration mimicking RP.17 Animals were examined at a time when the photoreceptor complement was reduced and it was found that retinal oxygen was elevated relative to control cats without retinal degeneration. Experiments were not directed at the retinal vasculature and, thus, no attempt was made to look at a vascular component of the pathology. These studies all support the hypothesis that photoreceptor loss, whether by laser application or inherited degeneration, leads to an increase in inner retinal oxygen tension. This can presumably result in altered cytokine levels, leading to vascular remodeling.

A recent report by Valter and colleagues18 described the relationship between photoreceptor cell death and tissue oxygen level in the RCS rat model of retinal degeneration. Because the primary defect in the RCS strain resides in the retinal pigment epithelial layer, the relevance of this model to human RP is unclear.19 However, the work warrants mention here because of its implications about the relationship between photoreceptors and tissue oxygen levels in more superficial retinal layers. Briefly, the authors reasoned that both healthy photoreceptors and photoreceptor debris in the subretinal space serve to limit diffusion of oxygen from the choroid to the inner retina. We add that the resulting hypoxia may explain the retinal neovascularization that has been widely reported in the RCS rat.20,21

Histopathologic studies of retinal tissue from humans with RP have revealed marked thickening of basement membrane around venules and capillaries.22 This work suggests that in the RP retina extracellular matrix deposits can, in extreme cases, occlude the vessel lumina, thereby compromising flow of nutrients and oxygen to the inner retina. We cannot comment on the occurrence of vessel occlusions of the type described by Li and coworkers,22 because occluded vessels would be expected to remain ADPase positive. Thus, occlusion cannot adequately explain the absence of ADPase-stained vessels in large regions of our transgenic retinas, nor would hypoxia be expected to reverse vessel occlusion. Of course, these discrepancies between human RP and phenotypic expression in transgenic mice may be due to the specific type of retinal mutation or to the difference in species. Still, retinal vessels have displayed normal regulatory responses to increased oxygen in patients with RP.23 This finding led to the conclusion that reduced retinal blood flow in RP is not due to vessel occlusion but to decreased metabolic load. It was postulated further, that loss of the oxygen-consuming photoreceptors might increase oxygen levels in the inner retina, leading to vasoconstriction and reduced blood flow in retinal vessels.23 Again, because ADPase histochemistry stained both the luminal and abluminal endothelial cell surfaces, vasoconstriction alone (like occlusion alone) cannot prevent staining. We consider the present work the first direct test of Grauwald’s hypothesis.25 Moreover, these findings extend the capacity of the retinal vasculature to respond to metabolic need in this model beyond simple autoregulation to atrophy or growth of new vessels.

Specifically, we believe that the vascular manifestations of the retinal degeneration described in this work represent an indirect effect of photoreceptor loss. As the photoreceptors die and disappear in RP, the retina thins considerably and its oxygen requirement is greatly reduced. The choroid remains macroscopically unchanged until advanced disease,24,25 and it has limited capacity for autoregulation.26 Therefore, retinal thinning secondary to loss of oxygen-consuming photoreceptors allows for oxygen diffusing from choroidal capillaries to penetrate into the inner retina. With the metabolic needs of the remaining retinal neurons met by this neighboring oxygen source, the retinal vasculature apparently attenuates by constriction of arteries and arterioles and by complete closure and atrophy of some venules and capillaries. The latter characteristic is most pronounced nearest the source of choroidal oxygen diffusion where the oxygen level is expected to be highest, in the deep capillary plexus. It is impossible to determine within the framework of this study whether deep capillaries were more profoundly affected than the more superficial vessels because they are more susceptible to oxygen-induced remodeling or because they are simply closer to the primary
source of oxygen. By postnatal day 26 in this mouse model of RP, the deepest (most scleral) remaining capillaries consisted of simple loops extending from the superficial vessels to the outer plexiform layer, which now resided adjacent to the epithelium. Low ambient oxygen not only arrested retinal capillary atrophy, but it stimulated new capillary growth in both the deep and superficial vascular beds. The architecture of these new vessels was surprisingly normal given the presumption that they must have developed by exclusively angiogenic, rather than vasculogenic, mechanisms. Vasculogenesis, or formation of capillaries from pluripotent mesenchymal precursors, is the predominant form of vessel growth during retinal development.27

The ILE 255/256 deletion transgenic mouse, because its retinal pathology has many features in common with human RP, constitutes a rational model for studies of the basic pathogenesis of the condition. In addition, the model has the potential to serve as an appropriate testing ground for cell transplantation therapy. In this context, consideration must be given to the integrity of the retinal vasculature, which while having an incredible capacity for recovery, is profoundly affected by the progressive dystrophy.

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References