The Role of Fas-FasL in the Development and Treatment of Ischemic Retinopathy

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PURPOSE. Define a role for Fas-Fasl in oxygen-induced retinopathy and explore the mechanism of pigment-epithelium-derived growth factor (PEDF) inhibition in this model.

METHODS. Seven-day-old mice C57BL/6j (B6), Fasl-defective (B6-gld), or Fasl-defective (B6-lpr) mice were exposed to 75% oxygen for 5 days (postnatal day [P]7–P12) and returned to room air. On day P17, vascular architecture was assessed microscopically after perfusion with FITC-dextran, and preretinal nuclei were quantified by PAS and hematoxylin staining. In some experiments, mice were treated intraperitoneally with PEDF. Vascular architecture and preretinal nuclei counts were compared with those in PBS-treated control animals.

RESULTS. Oxygen-induced retinopathy was significantly increased in Fasl-defective gld mice compared with wild-type B6 animals. This was manifested by an increase in the number of microaneurysms, neovascular tufts, and preretinal nuclei. PEDF treatment prevented retinopathy in B6, B6-gld, and B6-lpr mice.

CONCLUSIONS. Fas-Fasl interactions regulate the extent of oxygen-induced retinal neovascularization. The inhibition of neo-vascularization in B6 gld, and B6-lpr mice by PEDF suggests that Fas-Fasl interactions are probably not the mechanism for inhibition in this model. (Invest Ophthalmol Vis Sci. 2003;44: 1282–1286) DOI:10.1167/iovs.02-0478

The formation of new blood vessels is essential for growth and development. It requires the sprouting and migration of endothelial cells, as well as endothelial cell proliferation and capillary tube formation (differentiation). The growth of new vessels from preexisting blood vessels (neovascularization) is also a significant component of organ and tissue homeostasis. This response can be initiated by inflammation,¹ ischemia,² or local factor production³ and is an essential process in wound and tissue repair.¹ There are, however, instances in which angiogenesis can have pathogenic consequences. This is the case in the eye, where neovascularization is a major component of several ocular disorders, including diabetic retinopathy (DR), retinopathy of prematurity (ROP), and age-related macular degeneration (AMD). In these disorders, the growth of new vessels can impair vision and threaten quality of life. Ischemic retinopathies (retinal neovascularization) are the major cause of acquired blindness in young people. It is thought that these processes are driven by growth factors such as VEGF.⁴

ROP is a proliferative disease of premature infants that causes blindness from the aberrant growth of retinal blood vessels. It is thought that the hypoxia in the retina induced after hyperbaric oxygen treatment of these infants is the initiating factor. An animal model of this disorder has been used to characterize the roles of various angiogenic and antiangiogenic factors.⁵ Exposure to hyperoxia induces cessation of blood vessel growth, resulting in nonperfusion of areas of the retina. When animals are returned to normoxia, the retina responds by increased VEGF production and uncontrolled vessel growth.

Recent studies from our laboratory have shown that the presence of Fasl in the eye is an important barrier to both inflammatory cells⁶ and new blood vessel growth.⁷ The control of inflammation is a component of immune privilege in the eye, where Fasl induces apoptosis in Fas-¹ lymphoid cells that invade the eye in response to viral infection⁸ or corneal grafting.⁹ We have also found that Fasl, expressed by RPE cells, controls new vessel growth beneath the retina.⁵ The loss of Fasl expression in this region may be a predisposing factor in AMD, in which new vessels originating in the choroid penetrate Bruch’s membrane and localize beneath the retina.

Neovascularization can also be controlled by the antiangiogenic factor pigment-epithelium-derived growth factor (PEDF). PEDF is constitutively present in the cornea and vitreous² and is a major antiangiogenic component of these tissues. It can be induced in the retina by hyperoxia and downregulated by hypoxia. This suggests that it may play a role in ischemia-driven retinal neovascularization. In vitro, PEDF inhibited endothelial cell migration toward angiogenic inducers such as platelet-derived growth factor, VEGF, and interleukin-8. Recently, it has been demonstrated that PEDF prevents the growth of new blood vessels in a model of ischemia-induced retinopathy in mice.¹⁰ This study further suggested that the mechanism of inhibition by PEDF is through the induction of apoptosis. Recently, Fasl-Fasl interactions have been implicated in the mode of action of PEDF on angiogenesis.¹¹

In the current study, we examined the role of the Fas-Fasl in the development of ischemia-induced retinopathy, as well as the role of the receptor–ligand pair in the successful treatment of retinopathy with PEDF. Our results demonstrate that although Fasl and Fasl play a role in the evolution of the retinopathy, they do not appear to be involved in the mode of action of PEDF in the ROP model.

METHODS

Mice

C57Bl/6 (B6) mice were purchased from the National Cancer Institute (Frederick, MD). The B6-gld and B6-lpr mice were bred in our own facility from strains originally obtained from Jackson Laboratories (Bar Harbor, ME). Breeders are replaced once a year with new stocks.

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obtained from Jackson Laboratories. Mice are screened after the first generation of crosses with our colony, by using a PCR assay (described later). Each group contained five mice, and both eyes were evaluated in each subject. In most cases, one eye was used for histologic sections and one eye was evaluated by angiography.

Genotyping of lpr and gld Mice

Gld and lpr mice are screened by PCR. The gld screen uses two pairs of primers that are both run on isolated tail DNA: forward, A: 5'-GAACCCCACTCAG-3', reverse, B: 5'-CCGAATAAGCTCTTA-3'; forward, C: 5'-TAAAGACCTTTCCTGGA-3', reverse, D: 5'-TGAGGTGACTG- CATGC-3'. A single AB product of 377 bp denotes wild type; a single product of 650 bp from CD denotes gld. The appearance of both bands denotes a heterozygote. The lpr screen used three primers: forward primer A: 5'-TGACTTGCTCAGCACTCC-3', and reverse primers B: 5'-CGAGATCCTTTTCCTGGA-3' and C: 5'-GGCAGATCTGAGTAC-AGCAGCAG-3. A 495-bp produced from AB denotes lpr, an AC product of 247 bp denotes wild type. If both products are obtained, the mouse is heterozygous.

Induction of Retinopathy

Mice with their dams were exposed to 75% \( \text{O}_2 \) and 25% \( \text{N}_2 \) (premix; Airgas, Inc., St. Louis, MO) from postnatal day (P)7 to P12. Oxygen levels were monitored with an oxygen analyzer (Model D2; Beckman, Irvine, CA). After return to room air, the degree of retinopathy was determined by angiography on P12 and P17. Some animals were used to assess the degree of oxygen-induced retinopathy through histology on P12, P14, P17, P21, and P26. Normoxia control subjects were examined on the same days, and no retinal abnormalities or preretinal nuclei were observed under these conditions. Our study strictly followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Retinal Angiography

Mice were anesthetized with 0.1 mL/20 g with a ketamine-xylazine cocktail. The cocktail was made by mixing 1 mL of 100 mg/mL ketamine with 0.15 mL of 100 mg/mL xylazine, diluted 1:4 in saline before injection. Mice were then perfused through the left ventricle with a solution of 50 mg \( 2 \times 10^6 \) molecular weight FITC-dextran (Sigma, St. Louis, MO), in 1 mL phosphate-buffered saline (PBS). After enucleation and fixation in 4% paraformaldehyde for 24 hours, retinas were isolated, and flatmounts were created by performing three to four radial relaxing incisions. Fluorescence microscopic analysis was then performed to assess the degree of retinopathy.
Quantitative Histologic Evaluation

Neovascular retinopathy was determined by quantifying preretinal nuclei in histologic sections from eyes harvested on P17. They were fixed overnight in neutral buffered formalin, embedded in paraffin, and cut into 6-μm sagittal sections. Sections were stained with periodic acid Schiff base (PAS), and nuclei were quantified by light microscopic evaluation (magnification, ×400). Sections 30 to 90 μm apart obtained from both sides of the optic nerve were used. Sections that included the optic nerve were excluded to avoid counting errors. Endothelial cell nuclei on the vitreal side of the internal limiting membrane were counted as preretal.

Approximately 150 sections were counted per eye, as all sections did not contain preretinal nuclei.

Treatment with PEDF

Mice returned to room air on P12 were treated by daily intraperitoneal injection of 10 or 25 μg PEDF (BioProducts Maryland, Inc., Middletown, MD). Control animals were treated with PBS. Eyes were harvested from 75% O2, both strains showed typical signs of.

Effect of Treatment with PEDF

Recently, it was reported that treatment with PEDF prevents the development of retinopathy when treatments are given daily from P12 to P17 after hyperoxia.10–13 Inhibition by PEDF seemed to correlate with an increased number of apoptotic cells within the retina and a subsequent study determined that PEDF may function by Fas-FasL interactions in the cornea and in vitro.11 To assess the role of Fas and FasL in PEDF inhibition in ROP, B6, B6-gld, and B6-lpr mice were treated with 10 or 25 μg/d PEDF from P12 to P16. These doses have shown a significant effect in this model. Figure 4 demonstrates that treatment with 25 μg/d PEDF significantly reduced the numbers of preretinal nuclei, whereas treatment with 10 μg/d had no effect. Figure 5 shows representative histologic staining of untreated (Figs. 5A, 5C, 5E) and treated (Figs. 5B, 5D, 5F) B6, B6-gld, and B6-lpr mice on P17. In all strains, PEDF significantly

Figure 2. Quantification of preretinal nuclei. The total number of vascular nuclei extending from the internal membrane into the vitreous was counted on P12, P17, P21, and P26. *Significantly different from B6 (P < 0.01).

Figure 3. Preretinal nuclei in B6 (A) and B6-gld (B) mice on day 17 after PAS and hematoxylin staining. PAS and hematoxylin staining; magnification, ×400.

Figure 4. Inhibition of preretinal nuclei with PEDF. B6, B6-gld, and B6-lpr mice were exposed to hyperoxia from P7 to P12. They were then treated daily with 10 or 25 μg PEDF from P12 to P16. Eyes were harvested on P17 and stained with PAS and hematoxylin. Nuclei counts were then performed. *Significantly different from the PBS-treated control (P < 0.01).
the factors involved in these pathogenic processes.\textsuperscript{5,14,15} retinopathy. This model has been used successfully to study sim-
ilar to that observed in the human diseases ROP and diabetic Penetrated the vitreous cavity. This creates a retinopathy sim-
ilarly does not regulate development of the retinal vasculature, however, the neovascular disease was much worse in the FasL-defective animals. We observed that FasL expression on the RPE controlled the formation of new vessels induced by light injury. Thus, it appears that Fas-FasL interactions control the extent of neovascularization in the choroid and retina. It is important to note, however, that this interaction is not an absolute barrier, but is a mechanism to regulate the extent of the disease. It is noteworthy that FasL-defective mice have no obvious problems with the retina\textsuperscript{6-7,10} or choroidal vascula-
ture under normal conditions (not shown). Thus, FasL proba-
ably does not regulate development of the retinal vasculature, but is involved in controlling the extent of aberrant formation of new vessels induced by retinal ischemia. Other factors involving a balance between angiogenic and antiangiogenic factors are clearly important.\textsuperscript{14,15} As in the laser-induced CNV model\textsuperscript{7} we noted in the current model that retinal neovascularization in the B6\textsuperscript{lpr} and B6\textit{gld} mice did not always show the same degree of severity (see Fig.
4). Although we do not have an explanation for this, we can speculate that it is related to the incomplete knockout of Fas in the \textit{lpr}\textsuperscript{17,18} The increased severity of the lymphoproliferative syndrome in Fas-knockout mice compared with \textit{lpr} mice also supports this.\textsuperscript{19} We could hypothesize that the reduced Fas in the \textit{lpr} mouse coupled with normal levels of Fasl in the retina lead to increased inhibition (apoptosis) of vessel growth. The

### DISCUSSION

We used a murine model of ischemic retinopathy to examine the role of Fas and FasL in retinal neovascularization. In this model, neonatal mice are exposed to hyperoxic conditions for 5 days (P7–P12), during the time when the retinal vasculature is developing. This inhibits the development of vessels as the retina accommodates the high oxygen levels. When mice are returned to the relative hypoxia of room air, the retina becomes ischemic, and extensive neovascularization follows. The disease typically peaks 5 days after return to normoxia (day P17) and is exemplified by a significant number of vessels that have proliferated, crossed the inner limiting membrane, and penetrated the vitreous cavity. This creates a retinopathy similar to that observed in the human diseases ROP and diabetic retinopathy. This model has been used successfully to study the factors involved in these pathogenic processes.\textsuperscript{5,14,15}

We have examined the role of the apoptosis, inducing the molecule FasL and its receptor Fas, in this disease syndrome. Our data demonstrate a role for these molecules in some facets of the disease. A hallmark of this model in the mouse is the absence of retinal vessels around the optic disc after hypoxia, evidenced by a large area of nonperfusion on angiography. This seems to occur because the retinal vessels do not develop, as well as because of some loss of vessels from the hyperoxia. This process, observed on P12, was identical in B6 and B6\textit{gld} animals, suggesting that FasL plays no role in the process.

When the extent of neovascularization was assessed on P17, a significant role for Fas-FasL was observed. FasL-defective B6\textit{gld} mice had significantly increased disease, as determined by the increased number of preretinal nuclei, the elevated number of microaneurysms, and an increase in neovascular tufts. Thus, the apoptotic process mediated by Fas and FasL regulates the extent of neovascularization in this model.

When we assessed the resolution of the disease by monitoring preretinal nuclei until day 26, we observed that B6\textit{gld} and B6 mice had few preretinal nuclei and that this regressive phase followed the same kinetics in both strains. Thus, as in the initial phase of the disease, Fas-FasL interactions are not involved in the resolution of the disease.

The role of Fas-FasL in this model is similar to that observed in a model of choroidal neovascularization (CNV).\textsuperscript{7} In this model, angiogenesis was induced in wild type and B6\textit{gld} mice; however, the neovascular disease was much worse in the FasL-defective animals. We observed that FasL expression on the RPE controlled the formation of new vessels induced by light injury. Thus, it appears that Fas-FasL interactions control the extent of neovascularization in the choroid and retina. It is important to note, however, that this interaction is not an absolute barrier, but is a mechanism to regulate the extent of the disease. It is noteworthy that FasL-defective mice have no obvious problems with the retina\textsuperscript{6-7,10} or choroidal vascula-
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![Figure 5](https://example.com/image.png)

**Figure 5.** Representative histologic sections from PEDF-treated mice. Mice were treated with 25 µg PEDF from P12 to P16. Eyes were harvested on P17 and stained with PAS and hematoxylin. Retina of untreated (A, C, E) and treated (B, D, F) B6 (A, B), B6-gld (C, D), and B6-lpr (E, F) mice are shown. Arrowheads: preretinal nuclei. Magnification, ×200.
leakiness of the lpr mutation provides functional Fas and results in an imbalance in which the proportionally higher amount of FasL in the lpr cornea successfully controls formation of new vessels. This area must be studied further.

Recent reports have shown that PEDF can significantly reduce the extent of retinopathy in this model.10,11 To gain further insight into the mode of action of PEDF in the ischemic retinopathy model, we used Fas-defective lpr and FasL-defective gld mice. Our data clearly show that PEDF inhibited angiogenesis to a similar extent in B6, B6-gld, and B6-lpr mice. Thus, PEDF did not use Fas-FasL interactions to inhibit angiogenesis in this model. Thus, although PEDF-induced apoptosis through Fas-FasL may be the mechanism in some cases,11 this receptor–ligand pair are not involved in ischemic retinopathy. A role for other death receptors is possible (e.g., TNF-R and TNF-Related Apoptosis Inducing Ligand–Receptor [TRAIL-R]), but more study is necessary to determine their influence. It is also possible that the mode of action of PEDF in this model will rely more on its ability to prevent endothelial cell migration.9 Preventing migration of stimulated endothelial cells may lead to their death. Indeed, integrin connections generate antiapoptotic signals that promote endothelial cell survival.20 Failure to make such connections leads to apoptotic death that does not involve Fas-FasL.

The formation of blood vessels in the eye is an important component of several blinding disorders, including AMD, diabetic retinopathy, and ROP. The factors that regulate blood vessel growth are becoming known, but successful therapies for these sight-threatening disorders have been illusive. Understanding the molecules that induce neovascularization and gaining insight into the role of apoptosis in controlling angiogenesis are important in designing rational therapies for the treatment of these diseases.

References


