Increased Retinal Neovascularization in Fas Ligand–Deficient Mice

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PURPOSE. To investigate whether the absence of the Fas-Fas ligand system of apoptosis regulation affects hyperoxia-induced retinal vaso-obliteration and retinal neovascularization in a mouse model of oxygen-induced retinopathy.

METHODS. C57BL/6 (B6) and congenic Fas-ligand–deficient generalized lymphoproliferative disease (gld) mice were exposed to 75% oxygen from postnatal day (P)7 to P12 and then allowed to recover in room air. Eyes obtained from P7, P8, P10, P12, P14, P17, and P21, from both hyperoxia-injured and room air control animals were processed for histopathologic examination. Retinopathy was also qualitatively assessed in FITC-dextran perfused retinas by fluorescence microscopy. TUNEL assays were used to compare apoptosis in B6 and gld mice. Intraretinal blood vessel formation was quantitated by immunolabeling with an anti-type-IV collagen antibody. Retinopathy was further assessed by quantitation of preretinal neovascular nuclei on P17. RT-PCR was used to examine retinal expression of Fas and Fas ligand (FasL) over a time course of hyperoxia-induced retinopathy.

RESULTS. In hyperoxia-injured mice, the same degree of vasoobliteration was apparent on P8, P10, and P12 in B6 and gld mice. By P17, the hyperoxia-exposed FITC-perfused retinas of both strains exhibited preretinal neovascular tufts. However, P17 gld hyperoxia-exposed retinas exhibited approximately a 50% increase in preretinal neovascular nuclei compared with B6 mice. In addition, a subset of apoptotic cells located solely within the neovascular tufts on P17 were significantly decreased in hyperoxia-exposed gld retinas, compared with B6 control animals. RT-PCR showed an increase in the expression levels of Fas in both strains of mice as a result of hyperoxia-induced injury.

CONCLUSIONS. These data suggest that the Fas-FasL interaction plays an important role in retinal neovascularization after hyperoxia-induced injury. The absence of functional Fasl leads to an increased incidence of preretinal neovascular nuclei and decreased retinal apoptosis suggesting that this pathway may serve as a means of regulating endogenous endothelial cell populations in pathologic angiogenesis. (Invest Ophthalmol Vis Sci. 2003;44:5202–5210) DOI:10.1167/iovs.03-0050

Materials and Methods

Animals

C57BL/6 (B6) mice were originally obtained from Simonsen Laboratories (Gilroy, CA). Fas-L-deficient (gld) mice (on a C57BL/6 background) were obtained from Jackson Laboratories (Bar Harbor, ME).

Mice were housed and bred in the Oregon Health and Science University animal care facilities and treated in accordance with NIH guidelines and the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were provided food and water ad libitum and were kept on a 12-hour light–dark schedule. To induce retinopathy, postnatal day (P)7 B6 and gld mice, along with nursing females, were exposed to 75% oxygen for 5 days and then allowed to recover in room air on P12, according to the protocol of Smith et al. Room air control litters were maintained...
under identical conditions as the hyperoxia-exposed mice. The hyperoxia-exposed and room air control pups were killed by CO2 euthanasia or cervical dislocation on P8, P10, P12, P14, P17, and P21. Both eyes were carefully enucleated from each mouse, and one eye was placed in 10% neutral-buffered formalin overnight and routinely processed for paraffin embedding. These eyes were sectioned at 5-μm intervals, mounted on slides (SuperFrost Plus; Fisher Scientific, Pittsburgh, PA) and stored at room temperature until used for immunohistologic and TUNEL analysis. The retina of the contralateral eye was dissected for RNA isolation followed by RT-PCR analysis.

Retinal Fluorescein Angiography
Hyperoxia-exposed and room air control eyes were examined at P7, P8, P12, and P17 for qualitative assessment of the retinal vasculature by fluorescein angiography.21 The mice were deeply anesthetized by subcutaneous injection of a cocktail containing ketamine, xylazine, and acepromazine. Mice were then perfused through the left ventricle with 1 mL of PBS containing 50 mg high-molecular-weight (2,000,000), FITC-conjugated dextran (Sigma-Aldrich, St. Louis, MO). The animals were then killed by cervical dislocation and the eyes enucleated and placed in PBS. Cornea, iris, sclera, and RPE were dissected, and the lens were then killed by cervical dislocation and the eyes enucleated and placed in PBS. Cornea, iris, sclera, and RPE were dissected, and the lens and retina were fixed in 4% paraformaldehyde for 3 hours at 4°C. After fixation, the lens was removed and the retina was incised radially and flattened with an antifade mounting medium (SlowFade; Molecular Probes, Eugene, OR). The retinal vasculature was visualized by fluorescein microscopy and photographed with a digital camera.

Immunohistochemistry
A rabbit polyclonal anti-mouse type-IV collagen antibody (Collaborative Biomedical Products, Bedford, MA) was used to immunolocalize the basement membranes of blood vessels in the hyperoxia-exposed retinas as well as the age-matched room air control retinas. Before incubation with the type-IV collagen antibody, the sections were digested with 0.1% pepsin (Sigma-Aldrich) for 20 minutes at room temperature. The sections were rinsed with deionized water, and then washed with Tris-buffered saline (50 mM Tris, 0.15 M NaCl [pH 7.5]; TBS). Non specific binding sites were blocked with 2% normal goat serum (Vector Laboratories, Burlingame, CA), 0.1% BSA, and 0.3% Triton X-100 in TBS for 60 minutes at room temperature. The sections were incubated overnight at 4°C in a humidified chamber with the antitype-IV collagen antibody at a dilution of 1:400 (2.5 μg/mL). Sections were washed in TBS and incubated with a biotinylated goat anti-rabbit IgG antibody (1:200; Vector Laboratories) for 60 minutes at room temperature. The sections were washed in TBS, and the antibody-antigen complexes were visualized using fast red as the substrate (Vector Laboratories, Burlingame, CA). The tissue sections were counterstained with hematoxylin and covered with mounting medium (Crystal/Mount; Biomedica, Foster City, CA). Retinal vessels were quantitated in the following categories: (1) superficial vessels, defined as those located between the inner limiting membrane and the ganglion cell layer; (2) transitional vessels, defined as those connecting the superficial and deep vascular networks stretching between the inner plexiform and inner nuclear layers; and (3) deep vessels, defined as those located in the outer plexiform layer (n = 4–6 animals per time point, two sections per animal).

Neovascular Nuclei Quantification
To quantitate the retinal neovascularization, tissue sections were stained with hematoxylin and eosin (H&E). Retinal vascular cell nuclei anterior to the inner limiting membrane of gld and B6 hyperoxia-exposed and room air control retinas were counted at P17 in a masked fashion. Care was taken to avoid counting hyaloid vessel nuclei near the optic disc and lens, which are easily distinguishable from the neovascularization extending into the vitreous. The average neovascular nuclei per section per eye is calculated as the mean of 18 counted sections, 40 μm apart (n = 6–8 eyes).

TUNEL Assay
Multiple sections (n = 4–6 sections per mouse) on opposite sides of the optic nerve were selected from each eye at each time point (n = 4–6 mice per time point). Sections were deparaffinized with xylene and hydrated in graded concentrations of ethanol and TBS. A peroxidase in situ apoptosis detection kit (Apoptag; Intergen, Purchase, NY) was used to label exposed 3'-OH ends of DNA fragments in apoptotic cells, according to the manufacturer’s instructions. Apoptotic cells were visualized with DAB substrate and counterstained with methyl green to aid in the morphologic evaluation of the retinal cells. TUNEL-positive cells were counted in a masked fashion, and the counts were normalized to the number of positive cells per millimeter of retinal length. Furthermore, TUNEL-positive cells that were located exclusively within the neovascular tufts were counted and reported as a total number of tuft-positive apoptotic cells per section.

In addition, another in situ apoptosis detection kit (Apoptag Red; Intergen) was used for fluorescent labeling of apoptotic cells within FITC-perfused retinal wholemounts, for coordinated visualization of apoptotic cells and the retinal vasculature. Double-labeled wholemounts were visualized by both fluorescence and confocal microscopy and photographed with a digital camera.

Electron Microscopy
Electron microscopy (EM) was used to confirm the TUNEL technique as a valid assay for apoptosis in retinal tissue. Animals were fixed by intracardiac perfusion and the eyes enucleated and processed for EM as previously described.22 The sections were examined and photographed on an electron microscope (JEM-100CXII; JEOL, Peabody, MA).

RT-PCR Analysis
Retinas were dissected at selected time points and pooled for RNA extraction. Total RNA was isolated using RNA isolation solution (Stratagene, La Jolla, CA) and then reverse transcribed to obtain cDNA. Touchdown RT-PCR detection of gene expression was performed as previously described.23 Briefly, first-strand cDNA synthesis was performed with oligo(dT) primed Moloney murine leukemia virus (M-MLV) reverse transcriptase for 2 hours at 37°C (Life Technologies, Rockville, MD). cDNA was amplified with a denaturing step at 94°C (15 seconds) an initial annealing temperature of 69°C (1 minute), and a 2-minute extension at 72°C for two cycles. The annealing temperature was reduced by 1°C every two cycles until it reached 60°C. An additional 10 to 20 cycles with an annealing temperature of 55°C were run, depending on predetermined conditions. Mouse-specific Fas receptor primer sets (sense, 5’-GGGCTATGGTTGTTGACATC-3‘; anti-sense, 5’-AGCACAGGACACAGCTGGACTT-3‘); anti-FasL primer sets (sense, 5’-ACCACACACGCCCATCATA-3‘; anti-sense, 5’-GCGAGTCAGGCGTTCATA-3‘), all from Integrated DNA Technologies Inc. (IDT, Coralville, IA) were used to amplify specific cDNAs. A primer pair for the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was included in each assay as an internal control (sense, 5’-GATGGCCCTCCGTGTCCTA-3‘; anti-sense, 5’-GGGCCATGGAAGCTGGAAAGT-3‘; IDT). The PCR products were electrophoresed in 3% agarose gels in Tris-acetate buffer containing ethidium bromide and subsequently photographed under UV light.

RESULTS
Retinal Fluorescein Angiography
B6 and gld room air-raised and hyperoxia-exposed mice were perfused with FITC-dextran to label the retinal vasculature and visualize neovascular tufts. Room air gld mice had normal vascular development on P12 and P17 when compared with B6 control animals (data not shown). In addition, after 5 days of hyperoxia (O2) ending on P12, the mutant mice had the same vascular development on P12 and P17 when compared with B6 controls, and the mutant mice had the same vascular development on P12 and P17 when compared with B6 controls. A rabbit polyclonal anti-mouse type-IV collagen antibody at a dilution of 1:400 (2.5 μg/mL). Tissue sections were washed in TBS, and the anti-rabbit IgG antibody (1:200; Vector Laboratories) for 60 minutes at room temperature. The sections were washed in TBS, and the antibody-antigen complexes were visualized using fast red as the substrate (BioGenex Laboratories, San Ramon, CA). The tissue sections were counterstained with hematoxylin and covered with mounting medium (Crystal/Mount; Biomedica, Foster City, CA). Retinal vessels were quantitated in the following categories: (1) superficial vessels, defined as those located between the inner limiting membrane and the ganglion cell layer; (2) transitional vessels, defined as those connecting the superficial and deep vascular networks stretching between the inner plexiform and inner nuclear layers; and (3) deep vessels, defined as those located in the outer plexiform layer (n = 4–6 animals per time point, two sections per animal).

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and gld mice also qualitatively showed similar avascular regions on P17 after hyperoxia-exposure and 5 days’ recovery (Figs. 1C, 1D). Analysis of P17O2 retinas at high magnification revealed more extensive neovascular tufts in gld mice than in B6 mice (Figs. 1E, 1F, arrows). The tufts in the gld mice were often large sheets of preretinal cells as opposed to smaller “popcorn” tufts in the B6 mice. Furthermore, the retinal vessels in the gld mice appear to exhibit somewhat more dilatation than the vessels of the B6 mice. These qualitative findings were then confirmed quantitatively by examination of cross sections.

Type-IV Collagen

To compare and contrast further the retinal vascular response between the B6 and gld mice, 5-μm tissue sections were stained for type IV collagen. Type IV collagen is a major component of the basement membrane in retinal vessels and has been used as a marker of vessels in the developing retina. Immunolabeling for type IV collagen revealed retinal vessels present in the superficial (Fig. 2A, arrowhead), transitional (arrow), and deep (asterisk) layers of the retina in P12 B6 room air-raised mice. A similar staining pattern was seen in the P12 room air gld mice (Fig. 2B). Both types of hyperoxia-exposed mice had no transitional and deep retinal vessels on P12 (Figs. 2C, 2D). Similar to the P12 room air retinas, the P17 room air retinas expressed type IV collagen in all three defined layers, as detected by immunohistochemistry (data not shown). Staining of P17O2 retinas revealed a comparable redevelopment of the vascular networks in most of the retina in both the B6 (Fig. 2E) and gld mice (Fig. 2F). There was a slight delay in the development of the deep vascular network at the midperiphery (Fig. 2E, 2F) in both B6 and gld mice compared with room air control animals. The neovascular tufts that extended beyond the inner limiting membrane also stained positive for type IV collagen in the B6 (Fig. 2E, arrowheads) and gld mice (Fig. 2F, arrowheads). Vessels were quantified in the superficial, transitional, and deep vascular beds from both room air and hyperoxia-exposed B6 and gld mice, as described in the Methods section. There was no significant difference in the number of intraretinal vessels between the two types of mice after hyperoxia-induced injury (data not shown).

Retinal Vasculature during the Early Vaso-obliteration Phase

Because of the similar vascular patterns seen at P12O2, we examined the vasculature at earlier time points in both B6 and gld mice to determine whether hyperoxia causes retinal vessel obliteration or simply prevents vessel formation. FITC-perfused retinal wholemounts were analyzed on P7, P8, and P8O2 in B6 and gld mice (Fig. 3). Fluorescein angiography in gld mice demonstrated that only the superficial vascular bed was present on P7 (Fig. 3A) with no deep vasculature (Fig. 3B). Development of the deep vascular network occurred on P8 (Fig. 3D); note the blurry, out-of-focus, deep vasculature in the background while focused on the superficial network (Fig. 3C). Vascular development was altered by hyperoxia, with vaso-obliteration of capillaries located in the central retina (Fig. 3F).
3E) and prevention of deep vessel development in both the central (Fig. 3E) and peripheral regions of the retina (Figs. 3F, 3G). Similar results were observed in the B6 mice (data not shown). These results were confirmed by immunohistochemical staining at the same time points for type IV collagen in retinal cross-sections (data not shown). Although it appears that the high-oxygen environment delays the deep vascular network’s development, it is important to note that the superficial layer undergoes vaso-oblation in the central retina.

Quantification of Retinal Neovascularization

To assess quantitatively the extent of preretinal neovascularization, vascular nuclei anterior to the inner limiting membrane were counted at P17. As expected, no vessels in the room air control retinas extended into the vitreous (data not shown). Conversely, 100% of mice exposed to hyperoxia from P7 to P12 and recovered in room air until P17 developed neovascular tufts extending beyond the inner limiting membrane into the vitreous (Fig. 4). In the B6 P17O2 mice (n = 6 mice, 18 sections per eye), the average number of neovascular nuclei extending into the vitreous was 24.8 ± 2.7 per 5-μm retinal cross-section (Fig. 4A), compared with 38.8 ± 8.7 nuclei per 5-μm cross-section in gld mouse eyes (n = 6 mice, 18 sections per eye; Fig. 4B). The absence of Fas-Fasl-induced apoptosis resulted in a 50% (P < 0.03, unpaired t-test) increase in preretinal neovascular nuclei in the gld mice (Fig. 4C).

Apoptosis

To investigate the role of apoptosis further in our model, we examined an extended time course of both B6 and gld mice by the TUNEL method. The number of retinal apoptotic cells peaked on P17O2 in the B6 mice (13.9 ± 5.8) and is higher than in the room air–raised B6 control retinas (5.2 ± 1.6). The gld mice showed significantly (P < 0.05 by one-way ANOVA followed by the Newman-Keuls multiple comparison post hoc test) fewer apoptotic cells in both the hyperoxia-exposed animals (3.9 ± 1.4) and the room air control mice (2.1 ± 0.7) on P17 compared with B6 mice. Whereas most of the apoptotic cells were located in the nuclear layers of the retina, some TUNEL-positive cells were located within the neovascular tufts anterior to the inner limiting membrane. Quantification of these TUNEL-positive cells localized to the tufts also revealed more apoptosis occurring in the B6 mice (Figs. 5A, 5C) compared with gld mice (Figs. 5B, 5D). B6 mice averaged 2.44 ± 0.5 positive cells per section (n = 8 eyes, 12 sections per eye), whereas gld mice averaged 1.18 ± 0.26 apoptotic cells per section (n = 8 eyes, 12 sections per eye; P < 0.03, unpaired t-test). Morphologic examination by EM confirmed that the TUNEL-positive cells in the retinas were apoptotic and not necrotic (data not shown). The apoptotic cells were characterized by chromatin condensation, crescent-shaped spaces around the nucleus, and nuclear blebbing.

To characterize further the localization of apoptotic cells within the retina, TUNEL-positive cells were fluorescently labeled within FITC perfused retinal wholemounts and imaged by fluorescence and confocal microscopy. Three-dimensional images were compiled from confocal images acquired from different focal planes of the retina. En face imaging of representative retinal wholemounts revealed both vessels (Fig. 6A, green) and apoptotic cells (red) present throughout the retina. Rotation of the confocal image shows that most of the apoptotic cells are located in the nuclear layers of the retina (Fig. 6B, green) and apoptotic cells (red) present throughout the retina.
However, a few TUNEL-positive cells are located within the neovascular tufts (Fig. 6B, arrow), similar to the cross-sections. Further analysis of retinal cross-sections demonstrates that apoptosis in both strains of mice returned to room air levels by P21 (data not shown).

**RT-PCR Analysis**

The mRNA expression level of Fas and FasL during an extended time course further implicates the Fas-FasL pathway in this disease process. An increase in the expression of Fas was observed in B6 mice on P14, P17, and P21 after hyperoxia-induced injury and on P17 and P21 in gld mice (Fig. 7). A less consistent pattern of expression was noted for FasL in these mice (Fig. 7).

**DISCUSSION**

Previous animal studies of oxygen-induced retinopathy have demonstrated that hyperoxia results in retinal vaso-obliteration with subsequent ischemia-induced retinopathy when the animal is returned to normoxia. The vaso-obliteration has been shown to involve apoptosis of vascular endothelial cells (ECs). The Fas-FasL pathway can mediate EC apoptosis. Therefore, we initially hypothesized that the FasL mutant mice may have less EC apoptosis in a mouse model of hyperoxia-induced retinopathy and thus have reduced vaso-obliteration and subsequently less neovascularization during the room air recovery phase.

We initially compared the gld and B6 retinal vasculature after the period of vaso-obliteration (P12) and at the peak of neovascularization (P17). Vascular development was similar in room air control animals from both types of mice at P12 and P17, indicating that the absence of functional FasL did not appear to alter retinal vascular development. We also found no noticeable difference in central retinal vaso-obliteration after hyperoxia-induced injury. The amount of central vessel obliteration of the superficial vascular network remained the same even in the absence of functional FasL. These data imply that FasL may not play a role in the vaso-obliteration stage of oxygen-induced retinopathy. Alternatively, other apoptosis mediators, such as TNFα and TNF-related apoptosis-inducing ligand (TRAIL), may compensate for the loss of functional FasL.

We also observed that the deep vascular network was not present in either gld or B6 mice after the hyperoxia phase.
the deep vascular bed of the mouse retina does not develop consistent with a recent report by Fruttiger showing that the deep vascular network had not yet developed. This is mouse pups went into the high-oxygen environment (P7), (P12). During our studies, we observed that when the mouse pups went into the high-oxygen environment (P7), the deep vascular network had not yet developed. This is consistent with a recent report by Fruttiger showing that the deep vascular bed of the mouse retina does not develop until P8 and is also consistent with earlier reports of the presence of deep vessels in the outer plexiform layer at P8 to P10. Therefore, in addition to vaso-obliteration along the superficial vascular layer, the high-oxygen environment concurrently prevents the deep vessels from forming. The high-oxygen environment is known to decrease the levels of VEGF, and our results are consistent with VEGF’s acting as a survival factor for the existing superficial vascular network and an angiogenic factor for the deep vessels in both types of mice. However, the same degree of superficial obliteration occurs in the gld mice compared with the B6 control animals, which suggests that it is not a Fas-FasL-mediated process.

The most significant finding in this study was that the absence of the Fas-FasL apoptotic pathway resulted in an increase in preretinal neovascularization and overall reduced retinal cell death on P17O₂. Whereas most of these dying cells are found in the neuronal layers of the retina, a subset was observed within the neovascular tufts. In addition to the difference in total number of apoptotic cells between the B6 and gld mice, a significant difference was observed in the amount of apoptotic cells within the tufts of the B6 mice compared with the gld mice. ECs are a major cell type in the neovascular tufts as demonstrated by the presence of von Willebrand factor (vWF) immunoreactivity (Powers MR, unpublished observations, 2002). The increase in vascular tuft cells anterior to the inner limiting membrane in the gld mice on P17O₂ could be a direct result of reduced EC apoptosis in these mutant mice. In addition to the presence of ECs, we have shown that microglial cells are localized in the neovascular tufts on P17O₂ in the mouse model (Eubanks JP, Davies MH, Powers MR, ARVO Abstract 723, 2000). It appears that the ECs in the tufts are undergoing apoptosis, and the microglia could serve a phagocytic function or possibly even induce EC apoptosis. Previous studies have demonstrated that gial cells are absent from the neovascular tufts, as exhibited by negative GFAP staining, and are not a candidate for phagocytic function.

Pathologic angiogenesis is a complex process involving angiogenic growth factors, proteases, and adhesion molecules. ECs depend on survival factors (e.g., VEGF) to suppress apoptotic factors while stimulating intracellular molecules to promote cell survival. A change in the balance between angiogenic and antiangiogenic factors may lead to EC death. EC death has been demonstrated to occur through the Fas pathway in oxidized LDL-induced cell death, with an associated reduction in the cellular caspase inhibitor FLICE-inhibitory protein (FLIP). In addition, oxidized LDL-induced EC death was significantly reduced in aortic endothelium cultures from gld mice compared with B6 control mice, further supporting the role of Fas-FasL in EC apoptosis. In comparison to our results of increased preretinal neovascularization in gld mice in the model of hyperoxia-induced retinopathy, a prior study using a model of subretinal neovascularization also noted increased neovascularization in gld mice compared with B6 control animals. These investigators further showed that cultured choroidal ECs undergo apoptotic cell death through the Fas-FasL pathway when cultured with retinal pigment epithelial cells.

ECs have been shown to express both Fas and FasL, but are resistant to Fas-mediated apoptosis under normal conditions because of the inhibition of cell death by FLIP. However, EC Fas expression is upregulated under static and irregular flow conditions and ECs can undergo autoregulatory apoptosis through the Fas-FasL pathway under such conditions. Abnormal flow conditions are probably present in the preretinal tufts, which sometimes are not completely luminized. Fas expression is also increased on ECs when treated with growth
factors such as VEGF and bFGF in vitro.\textsuperscript{26} We saw an upregulation at the mRNA level of Fas in the P17O\textsubscript{2} retina, possibly a result of static and irregular flow conditions in the neovascular tufts. The ECs located within the neovascular tufts are probably primed for Fas-mediated cell death, because of the initial increased levels of VEGF in the hypoxic retina, but are initially resistant. However, by P17O\textsubscript{2}, waning levels of VEGF could tip the balance toward the upregulation of proapoptotic factors in conjunction with reduced levels of antiapoptotic factors such as FLIP.\textsuperscript{35} Mature vessels are likely to be resistant to this cell death through cell-cell and cell-matrix interactions.\textsuperscript{36} These findings suggest that apoptosis may play an important role in autoregulation of neovascularization and, without this level of regulation vessel growth, can proceed unchecked. As shown by Griffith et al.,\textsuperscript{35} the expression of FasL throughout the retina may be important for this regulation. Recently, it has also been demonstrated that EC apoptosis induced by thrombospondin-1 (TSP1) and pigment epithelium-derived factor (PEDF) occurs through the Fas-FasL pathway.\textsuperscript{26} In a model of corneal angiogenesis, neovascularization was inhibited by TSP1 and PEDF in B6 mice, whereas gld mice were resistant to this inhibition. PEDF has also been shown to be antiangiogenic in the mouse model of oxygen-induced retinopathy, adding credence to our hypothesis that preretinal EC apoptosis is Fas-FasL mediated.\textsuperscript{37}

Another explanation for the observed difference in retinal neovascularization, in addition to the upregulation of Fas, could be a change in expression of negative regulators of Fas-mediated apoptosis, such as Bcl-2\textsuperscript{38} or FLIP.\textsuperscript{32,38} Downregulation of these antiapoptotic proteins may result in increased incidence of FasL induced apoptosis within the preretinal tufts during hyperoxia-induced retinopathy, which results in more neovascularization and less apoptosis in the gld mice compared with the B6 mice. Future experiments are needed to elucidate the role that antiapoptotic factors may play in this model. However, because the Fas-FasL apoptotic path-

\textbf{FIGURE 5.} TUNEL assays of representative sections showing apoptotic cells in B6 and gld mice. P17 room air controls from B6 and gld mice show minimal to no apoptosis (data not shown). Increased neovascular tuft apoptosis was observed in oxygen-exposed B6 mice (A, C) compared with oxygen-exposed gld mice (B, D) on P17. Arrows: positive apoptotic cells (black) located within neovascular tufts; arrowheads: tufts without apoptotic cells. Magnification, $\times400$.

\textbf{FIGURE 6.} Confocal imaging of retinal wholemounts in P17O\textsubscript{2} gld retinas. En face image of the retina shows apoptotic cells (red) scattered throughout the retina (A). A 75° rotation of the image shows that most of the apoptotic cells are situated below the vascular beds of the retina in the outer nuclear layer (B, arrow). However, some TUNEL-positive cells are located within the neovascular tufts (B, arrow).
way is implicated in oxygen-induced retinopathy, these results suggest that the upregulation of FasL, or other EC specific proapoptotic factors may provide a new avenue of therapy for retinal neovascularization by promoting regression of vascular tufts.

In addition, the amount of neuronal damage incurred by hypoxic injury in this model has not been fully appreciated. Neuronal remodeling may occur through the Fas-FasL apoptotic pathway, as indicated by the reduction of non–vascular-cell death in the gld retinas. This neuronal injury could contribute to poor visual outcome, which correlates with the altered electroretinograms that are observed after ischemia-induced retinal injury. More specifically, there was a reduction in the amount of apoptosis within the tufts and an increase in the expression of the Fas receptor in the retina. These data suggest a role for Fas-FasL-mediated apoptosis in the regulation of retinal neovascularization and provide a novel approach for possible therapeutic treatment. Additional functional studies are needed to confirm the biological role for the Fas-FasL pathway in neovascular tuft remodeling and to determine the apoptotic pathways involved in the vaso-oblitration phase of ROP.

References


