Peroxisome Proliferator-Activated Receptor-γ Ligands Inhibit Choroidal Neovascularization

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ger-related macular degeneration (AMD) is the leading cause of visual loss in persons more than 65 years of age.1 The exudative form of the disease, characterized by choroidal neovascularization (CNV),2-3 is thought to be responsible for most of the cases of severe visual loss in this disease. In CNV, neovascular channels grow from the choroidal vasculature and extend into the subretinal space leading to local tissue damage.2 This is associated with activation and migration of the normally quiescent retinal pigment epithelial (RPE) cells into the stroma of the CNV lesion.4 Experimental and pathologic studies suggest a critical role for vascular endothelial growth factor (VEGF) in the pathogenesis of CNV and demonstrate that the activated RPE cell is a major source of this angiogenic cytokine.5-8 Laser photocoagulation of CNV membranes is currently the only widely accepted treatment modality. Novel therapies directed at preventing the development of CNV are urgently needed.3

Thiazolidinediones (TZDs) are a novel class of drugs that can be used to improve insulin resistance in non-insulin-dependent diabetes mellitus (NIDDM).9,10 Several members of the TZD class, including troglitazone (TRO) and rosiglitazone (RSG, formerly BRL 49653), have been or are currently available for clinical use in the treatment of NIDDM.11,12 TRO and RSG are synthetic high-affinity ligands for peroxisome proliferator-activated receptor (PPAR)-γ, a nuclear hormone receptor that is a member of the PPAR family.13-15 PPAR-γ acts by forming a heterodimer with the retinoid X receptor and binding to direct repeats of hormone response elements.10,16 PPAR-γ is expressed most abundantly in adipose tissue, where it promotes adipocyte differentiation and regulates the expression of genes involved in fatty acid metabolism.10,17 Recent studies have shown a more widespread distribution of PPAR-γ receptors, suggesting that PPAR-γ ligands may have effects in other tissues.17-19 These actions include inhibition of inflammatory processes in macrophages,20-22 neoointima formation after vascular balloon injury,23 and growth of cancer cells.24-26 The localization of PPAR-γ in specific retinal cell types has not been previously determined.

Increasing evidence suggests that PPAR-γ ligands have direct effects on endothelial cells (ECs). ECs express PPAR-γ mRNA and protein,29,30 and PPAR-γ ligands inhibit growth
factor-induced proliferation of ECs, increase plasminogen activator inhibitor (PAI)-1 expression, and suppress endothelin-1 secretion by ECs. PPAR-γ ligands inhibit growth factor-induced tube formation in human umbilical vein endothelial cells (HUVECs) and reduce VEGF receptor (VEGF-R) expression. The mitogen-activated protein kinase (MAPK) pathway is activated in ECs after stimulation by VEGF and may play an important role in cellular migration. PPAR-γ ligands inhibit MAPK-dependent migration of smooth muscle cells and may similarly inhibit this pathway in ECs. Most recently, data supporting an antiangiogenic effect of a PPAR-γ ligand (15-deoxy-Delta12,14-prostaglandin J2 [15d-PGJ2]) in vivo were provided by using a short-term VEGF-pellet corneal micropocket assay.

Laser photoagulation has been used successfully in animals to produce retinal lesions similar to those of human CNV. In both human and experimental CNV, a subretinal membrane forms that is composed of choroidal ECs (CECs) and RPE cells, is associated with local endogenous expression of VEGF, and leaks fluorescein for an extended period. In this study, both CECs and RPE cells expressed PPAR-γ and were inhibited in their response to VEGF by PPAR-γ ligands. In this well-established model of a chronic neurovascular disease, intraocular administration of the PPAR-γ ligand TRO had a prominent antiangiogenic effect on the development of CNV, without apparent toxicity to the adjacent retina.

**METHODS**

**Isolation of CECs and RPE Cells**

RPE cells were isolated from human fetal eyes (>22 weeks of gestation), which were obtained from the Anatomic Gift Foundation (Woodbine, GA), as previously described. Epithelial origin was confirmed by immunohistochemical staining for cytokeratin using a pan-cytokeratin antibody (Sigma, St. Louis, MO). No contaminating macrophages (anti-CD11; Sigma) or ECs (anti-von Willebrand factor; Sigma) were found.

CECs were isolated from bovine eyes for functional assays and Western Blot and from human fetal eyes for RNase protection assays (RPA). Cells were confirmed to be vascular ECs by positive immunostaining for von Willebrand factor and by binding of di-acylated low-density lipoprotein (LDL). Epithelial contamination was excluded by staining for cytokeratin. The CECs were isolated using magnetic beads carrying specific endothelial markers, as previously described. Lycopersicon esculentum (Sigma) was used for isolation of bovine CECs, and anti-CD31 antibody (Dako, Carpinteria, CA) was used for isolation of human CECs.

**RNase Protection Assays**

Antisense RNA probes were prepared from pGEM-3 cloning vectors containing partial cDNA probes for PPAR-γ kindly provided by Jeffrey Flier, Harvard University, Cambridge, MA). RPA vectors were linearized with either HindIII or EcoRI to prepare antisense probe and sense RNA reference, respectively. Antisense probes and sense RNAs were transcribed in vitro from linearized template DNA using bacteriophage T7, Sp6 (Promega, Madison WI), or T3 (Stratagene, La Jolla, CA) RNA polymerases. Antisense probes were used in an RPA to detect PPAR-γ and -α based on the sizes of protected bands: human PPAR-γ1, 258 bases; human PPAR-γ2, 348 bases; human retinoid X receptor (RXR)-α, 101 bases; and human reduced glyceraldehyde-phosphate dehydrogenase (GAPDH), 96 bases. GAPDH was included as a housekeeping mRNA in RPA to confirm the integrity of RNA in different samples.

For RPA, [32P] UTP-labeled RNA probes were incubated with 10 μg RNA in a final volume of 25 μl of hybridization solution for 12 to 16 hours at 56°C. Samples were then incubated for 1 hour at 45°C with a mixture of RNase A (90 μg/ml) and RNase T1 (600 U/ml). Nucleases were inactivated by treating samples with 10 mg/ml proteasine K for 1 to 2 hours at 37°C. After precipitation with ethanol, samples were resuspended in 7 μl gel-loading buffer (25% Ficoll, 0.2 M EDTA [pH 8], 0.25% bromophenol blue, 0.25% xylene cyanol). Samples were then electrophoresed on 6% nondenaturing polyacrylamide gels at 200 to 250 V for 2 hours. After electrophoresis, gels were dried and protected bands visualized by autoradiography.

**Western Immunoblot Analysis**

Protein extracts from CECs and RPE cells grown in vitro were lysed in a buffer containing 50 mM sodium pyrophosphate (pH 7.4), 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, and 100 μg/ml leupeptin before they were flash frozen in a dry ice and ethanol bath. After thawing, cells were scraped off the dish and centrifuged at 14,000 rpm. Nuclear and cytosolic fractions were prepared by the method of Dignam et al. Protein concentrations were determined using the Bradford assay. Full-length in vitro translated PPAR-γ1 was used as a positive control. Equal amounts of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using a transblotter (Bio-Rad, Richmond, CA). Nonspecific binding was blocked by using 5% fat-free milk powder and 0.1% Tween 20 in Tris-buffered saline. Membranes were incubated with a rabbit polyclonal anti-PPAR-γ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1:1000 for 2 hours in 0.2 M Tris-HCl (pH 7.5), 0.5 M NaCl buffer containing 5% fat-free milk powder and 0.1% Tween 20. Blots were washed and incubated for another hour with a goat anti-rabbit horseradish peroxidase–conjugated antibody (1:500) before final development using the chemoluminescent detection (Lumi-Glo; Kirkegaard & Perry, Gaithersburg, MD).

**Effect of TRO and RSG on Cell Proliferation**

To determine the effect of PPAR-γ ligands on proliferation of CECs, a [3H]thymidine uptake assay was performed in the presence or absence of TRO or RSG (0, 0.1, 1, 10, and 20 μM). Each experiment was performed in quadruplicate and repeated three times.

**Effects of TRO and RSG on Migration**

CEC and RPE cell migration was examined in cell culture chambers (Transwell; Costar Cambridge, MA). The polycarbonate membrane insert (with 8-μm pores) was coated with fibronectin (5 μg/well). Fifty thousand cells were added to the upper compartment of each well, and cells were then incubated at 37°C (95% air-5% CO2). Chemotaxis was induced by the addition of VEGF (10 ng/ml) to the lower compartment in the presence or absence of TRO or RSG (0, 0.1, 1, 10, and 20 μM). After 6 hours, the filters were washed with PBS and fixed with methanol (10 minutes at 4°C), followed by counterstaining with hematoxylin. The number of CECs and RPE cells per
field that migrated to the lower surface of the filters was determined microscopically (four fields per filter). Experiments were performed in triplicate and were repeated at least three times.

**Effect of TRO on Capillary-Like Tube Formation of CECs**

Three-dimensional tube formation was measured as previously described. A collagen mixture was placed in 24-well plates and incubated at 37°C for 3 hours to form gels. After polymerization of the gels, 1.0 × 10^5 CECs were seeded on each gel and incubated with endothelial basal medium (EBM) with 2% FBS overnight. The medium was then aspirated, and the remaining cells were overlaid with more gel (120 μl/well). EBM (1.2 ml) with 2% FBS, VEGF (10 ng/ml), and TRO (0–20 μM) was added to each well, and the medium was changed every 2 days. To evaluate the lengths of the capillary-like tubes in the gels, photographs were taken on day 5 with a phase-contrast microscope (nine random ×10 fields). The lengths of the tubes were measured with an image analyzer (Cosmozone 15; Nikon, Tokyo, Japan) and were expressed as an average length ± SD per well.

**Effect of Intravitreal Injection of TRO on Development of CNV Induced by Photocoagulation**

Thirty male pigmented rats (Brown Norway) and four cynomolgus monkeys were used. All procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Southern California Institutional Care and Use Committee. The rats were anesthetized with ketamine hydrochloride (45 mg/kg) and xylazine hydrochloride (10 mg/kg). The monkeys were anesthetized with ketamine hydrochloride (45 mg/kg), acepromazine maleate (0.005 mg/kg), and atropine sulfate (0.03 mg/kg). In rats, nine burns of diode laser photocoagulation (75-μm spot size, 0.1-second duration, 100 mW) were delivered to the retina, lateral to the optic disc through a slit lamp using a coverglass as a contact lens. Only lesions in which subretinal bubble or focal serous detachment of the retina developed were used for experiments. Fifteen rats received intravitreal injection of TRO (25 μM in 20 μl dimethyl sulfoxide [DMSO]), on days 1, 3, and 7, whereas the remaining 15 rats received only vehicle. In monkeys, seven photocoagulation burns (75 μm spot size, 0.1-second duration, 300 mW) were delivered in the paramacular region. Two eyes received intravitreal injection of TRO (250 μM in 100 μl DMSO) immediately after photocoagulation, whereas two other eyes received only vehicle. In the monkeys, a single injection of TRO was given to decrease the chance of traumatic cataract formation. CNV formation was detected as fluorescein leakage from the photocoagulation lesions in fluorescein angiography at 2 weeks (rats) and at 3 months (primates).

**Quantitation of Fluorescein Angiograms**

The effect of drug treatment was evaluated by semiquantitative assessment of late-phase (100–140 seconds after dye injection in rats and 5–5.5 minutes after dye injection in monkeys) fluorescein angiography, as previously described. Leakage was defined as the presence of a hyperfluorescent lesion that increased in size with time in the late-phase angiogram. Angiography was graded in a masked fashion by two examiners using reference angiograms. When the two scores for a lesion did not coincide, the higher score was used. Discrepant scoring was observed on only 12 of 142 lesions and was never greater than one grade. Angiograms were graded as follows: 0, no leakage; 1, slight leakage; 2, moderate leakage; 3, prominent leakage.

**Histopathologic Study of the Retina**

Fourteen days after photocoagulation, the rats were killed and eyes were enucleated and fixed in 0.2% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS (pH 7.4). Tissue samples were dehydrated and embedded in paraffin. Ten 3-μm serial sections were cut perpendicular to the retina from the central part of the lesion exhibiting the thickest laser-induced retinal destruction. The maximal thickness of the lesions from the disrupted RPE layer to the innermost aspect of the lesion was measured using an objective micrometer. In primates, 3 months after laser photocoagulation, one eye was fixed with 0.2% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS (pH 7.4) immediately after enucleation. The dissected macular regions were dehydrated in a series of graded alcohols and embedded in glycol methacrylate. Two- to three-micrometer sections were stained with periodic acid–Schiff.

**Electroretinograms**

Electroretinograms (ERG) were obtained for all primate eyes at baseline, and soon after photocoagulation and TRO injection. The pupils were dilated with topical 1% tropicamide sodium and 2.5% phenylephrine hydrochloride followed by dark adaptation for 30 minutes. Scotopic and photopic ERG were then obtained.

**Statistical Evaluation**

In Vitro Studies. In all analyses the normality for each group of numbers was tested using the Shapiro–Wilk statistic. Independent-sample t-tests were used to compare the control group and each of the TRO- and RS5-treated groups with the VEGF treatment group. Because four treatment groups were compared with the VEGF group, the accepted level of significance for all tests was α = 0.05/4 = 0.0125.

In Vivo Studies. The score per lesion and the percentage of lesions with leakage were calculated for each animal. Independent-sample t-tests were used to compare mean score per lesion, percentage leakage, and thickness between the TRO and control groups. Statistical significance was defined for α < 0.05.

**RESULTS**

**Expression of PPAR-γ in Retinal Cells Involved in CNV**

To determine whether PPAR-γ is expressed by cultured human RPE cells and CECs, we analyzed RNA from these sources using a sensitive RPA. As shown in Figure 1, human CECs and RPE cells exclusively express PPAR-γ1 mRNA as is evidenced by the single protected band of 258 bases detected by RPA. In contrast, both PPAR-γ1 and -γ2 (348-base protected band) mRNAs were expressed in untreated and phorbol 12-myristate 13-
acetate (PMA)-stimulated human monocytes. Significantly longer exposures failed to reveal the expression of PPAR-γ in CEC or RPE. Both RPE cells and CECs expressed mRNA for RXR-α (101-base protected band), a heterodimeric partner of PPAR-γ.

Expression and Subcellular Localization of PPAR-γ

To detect the expression of PPAR-γ protein in bovine CECs and human RPE cells, we performed Western immunoblot analysis of nuclear and cytosolic proteins. The data in Figure 2 reveal a band of 52 kDa corresponding to PPAR-γ1 that localizes almost exclusively to the nuclear fraction.

Effect of PPAR-γ Ligands on Thymidine Incorporation

The identification of increased numbers of CECs and RPE cells in CNV membranes suggests that proliferation of these cell types has occurred, and VEGF has been implicated as the growth factor mediating this effect. Functional VEGF-Rs are predominantly localized to ECs but have also been identified on RPE cells. VEGF (10 ng/ml) induced a 2.5-fold increase in thymidine incorporation in CECs (P < 0.0001) but had a more modest effect on RPE cells (P < 0.05), as previously reported (Fig. 3). TRO inhibited VEGF-induced thymidine incorporation in CECs and RPE cells in a dose-dependent manner beginning at 10 μM. Viability, measured by trypan blue exclusion, was greater than 95% at all drug concentrations tested. Addition of RSG inhibited proliferation of CECs and RPE cells in a dose-response manner at 1 μM of RSG and above (Fig. 4). Viability was greater than 95% at all drug concentrations tested.

PPAR-γ Activation Inhibits VEGF-Induced Migration of Retinal Cells

Migration of CECs and RPE cells into the CNV membrane plays an important role in the development of the lesion. VEGF (10 ng/ml) induced chemotactic migration of CECs and RPE cells in a modified Boyden chamber assay (P < 0.0001; Fig. 3). TRO significantly inhibited migration of both cell types (P < 0.0001) at 10 μM and higher for CECs and at 1 μM and higher for RPE cells. Viability was maintained at more than 95% at all drug concentrations tested.
concentrations used in this experiment. RSG also significantly inhibited migration of CECs and RPE cells; however, it was effective at concentrations of 1 μM and higher (Fig. 4).

**PPAR-γ Activation Inhibits VEGF-Induced Tube Formation of ECs**

Assembly of ECs into tubes is required for the formation of new vessels. VEGF (10 ng/ml) induced tube formation by CECs in a three-dimensional assay (P < 0.001; Fig. 5). Histologic confirmation of tube formation was obtained by microscopic examination of frozen sections of the gels (results not shown). Length of the tubes was measured with and without TRO in the presence of VEGF after 5 days of growth. TRO prominently inhibited tube formation at 10 μM (P < 0.001). At 20 μM, essentially no tube formation occurred. No morphologic evidence of cell death was observed.

**TRO Inhibits CNV in Rats**

Laser-induced CNV represents a reproducible and clinically relevant angiogenesis model to study CNV formation in the mature eye of both rodents and primates. In this model, new vessels grow from pre-existing choroidal vessels into the subretinal space in a manner similar to that found in neovascular AMD. The identification of local VEGF expression in this laser model suggests that mechanisms similar to those found in several forms of human retinal neovascularization are involved.39

When the retina and choroid of pigmented rats was exposed to intense laser photocoagulation, CNV developed in approximately 50% (34/64) of laser spots, determined by the presence 2 weeks later of well-defined lesions showing late-phase angiographic leakage of fluorescein dye (Fig. 6). Rats treated with intravitreous injection of TRO (20 μM) on days 1, 3, and 7 showed approximately 70% inhibition in the number of leaking lesions that developed (P < 0.0001; Table 1). The average leakage score per lesion was also significantly less in the TRO-treated animals (P < 0.0001).

Quantitative morphometric assessment of the CNV lesions revealed an average lesion thickness of 53.18 μm in the control lesions with a significant decrease in thickness to 25.0 μm in the TRO-treated group (P < 0.001; Fig. 7, Table 1). Morphologic examination of the lesions showed that the TRO-treated lesions were thinner, because they contained fewer vascular channels and RPE stromal cells (Fig. 7).

**Effect of TRO in Primate Retina**

Histologic assessment of paraffin sections of rat eyes showed no abnormalities in regions away from the laser lesions (results
not shown). Rat and primate eyes differ significantly in their structure because of the greater prominence of cones and the presence of a well-developed macula in the primate. Toxicity studies were therefore also performed in the primate. Acute toxicity was assessed by ERGs performed just after treatment with TRO; no functional retinal abnormalities were identified when compared with untreated controls (results not shown). ERGs were not performed at later time points. To assess the impact of TRO on neovascularization in primates, four eyes were treated with intense laser burns to induce CNV, and two of the eyes received TRO immediately after photocoagulation in late-phase fluorescein angiograms. In control eyes, the percentage of lesions showing leakage was similar in each group; however, the fluorescein leakage score per lesion was much

<table>
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<th>Table 1. Results of Late-Phase Fluorescein Angiography</th>
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<td><strong>Score/Lesion</strong></td>
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<tr>
<td><strong>Rat Eyes</strong></td>
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<td>Control (n = 10)</td>
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<td>Troglitazone (n = 12)</td>
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<td><strong>Monkey Eyes</strong></td>
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<td>Control (n = 2)</td>
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<td>Troglitazone (n = 2)</td>
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Late-phase fluorescein angiograms were performed on rats and monkeys (at 2 weeks and 3 months, respectively) after induction of CNV by photocoagulation. Control animals (treated with vehicle) were compared with those animals treated with intravitreal troglitazone. Leakage was defined as the presence of a hyperfluorescent lesion that increased in size with time in the late-phase angiogram. The table shows the percentage of photocoagulation lesions that showed leakage, as well as the average score per lesion (grades 1-4) when compared with standardized fluorescein angiograms. The maximal vertical thickness of the lesions was quantified histologically with an objective micrometer. Histologic measurements were not available in the monkey eye lesions. Data are means ± SD.

* Sample size for thickness measures, n = 4 control group, n = 5 troglitazone group.
† Independent sample t-test.

**FIGURE 6.** TRO inhibits leakage of fluorescein dye in laser-induced CNV in the rat. The retina and choroid of pigmented rats was exposed to intense diode laser photocoagulation. Nine lesions were placed in each retina. Animals were treated with intravitreous injection of TRO (20 mM) on days 1, 3, or 7 or with vehicle on the same schedule. Fluorescein angiography was performed 2 weeks after photocoagulation. (A) Three leaking lesions in the control retina (arrows); (B) absence of leakage in the lesions placed in the TRO-treated animal. Quantitation of these results is shown in Table 1.

**FIGURE 7.** TRO decreases the thickness of laser-induced CNV membranes in the rat. Lesions were generated as indicated in Figure 5. Two weeks after induction of CNV, lesions were dissected and embedded in paraffin. Sections through the thickest region of the membrane were obtained and the thickness of membrane measured (vertical bars). (A) Control CNV membrane in which neovascular channels (arrows) and intervening stromal cells are easily identified. (B) TRO-treated animals showed significantly thinner CNV membranes. The membranes appeared to contain fewer neovascular channels and fewer stromal cells. Horizontal bar, 25 μm.
whereas in mals show prominent fluorescein leakage from the laser spots, (5.25 minutes) fluorescein angiography at 3 months. fluorescein leakage from the photocoagulation lesions in late phase two other eyes received only vehicle. CNV formation was detected as fluorescein leakage. Quantitation of these data is shown in Table 1.

DISCUSSION

The exudative form of AMD, characterized by the presence of CNV, is responsible for most cases of severe visual loss in this common disorder. Use of destructive or vaso-occlusive therapies such as submacular surgery or laser photocoagulation is helpful, but in most patients these procedures are not indicated or are not sufficiently effective and the loss of vision progresses. An alternative but complementary treatment approach is to target the growth factor signaling pathways involved in CNV. Using this approach, CNV formation could be prevented by inhibiting the cells producing the angiogenic growth factors (i.e., the RPE), or by blocking the effect of the growth factor on the CECs.

PPAR-γ has very recently been recognized as a potential target for the pharmacologic therapy of pathologic neovascularization. PPAR-γ mRNA and protein are expressed by HUVECs, and their activation by PPAR-γ ligands inhibits the differentiation of HUVECs into tubelike structures. Furthermore, PPAR-γ ligands inhibit VEGF-induced angiogenesis in rat cornea.

We demonstrated the expression of PPAR-γ and its heterodimeric partner RXRα in RPE and in ECs derived from the choroid. The PPAR-γ gene produces two mRNA species by alternative promoter usage, each containing different 5’ exons that are spliced onto common downstream exons. PPAR-γ2 differs from -γ1 by the presence of 30 additional amino acids at its N terminus. Although both isoforms are expressed in abundant levels in human adipose tissues, PPAR-γ1 expression is typically much higher than PPAR-γ2 in nonadipose tissues. Consistent with these findings, both CECs and RPE cells express only PPAR-γ1 mRNA. At this time, the functional differences between the two isoforms are unknown.

PPAR-γ can be activated by poorly defined, naturally occurring ligands, or by members of the TZD class of drugs. The antidiabetic action of TZDs appears to be primarily mediated through activation of PPAR-γ; however, TRO is distinguishable from other TZD PPAR-γ ligands, because it also contains an α-tocopherol moiety which could have effects due to its antioxidant properties. Therefore in this study, in vitro experiments were also performed using RSG, a TZD that has no α-tocopherol moiety.

Neovascularization is a multistep process that includes degradation of basement membrane and proliferation, migration, and tube formation by ECs; the process is stimulated by a variety of growth factors and cytokines. VEGF plays a prominent role in the induction of ocular neovascularization and has been identified, and localized to RPE cells in surgically excised CNV membranes. In the retina, high-affinity VEGF-Rs are predominantly localized on ECs. Recently, functional KDR and Flt-1 VEGF-Rs have also been identified on RPE cells, and because RPE cells are a major source of VEGF in exudative AMD, it has been suggested that VEGF may also be an autocrine growth factor for RPE cells in AMD, as well as a paracrine factor for the choroidal endothelium. TRO and RSG both induced a strong dose-dependent inhibition of endothelial cell growth and migration in response to VEGF, providing strong support for the contention that this effect is mediated through PPAR-γ. Similar to previous studies concerning the effects of basic fibroblast growth factor (bFGF) on smooth muscle cell proliferation, RSG demonstrated stronger antiproliferative effect than TRO at lower concentrations (1 µM), consistent with its stronger binding affinity to PPAR-γ. The additional benefit of TRO over RSG, seen at concentrations of 10 µM and above, could be due to other non-PPAR-γ-mediated mechanisms that have been observed recently in other cell types. TRO contains a vitamin E (α-tocopherol) moiety, and we have previously shown that vitamin E (α-tocopherol) succinate inhibits both the proliferation and migration of RPE cells in vitro. The effect of higher concentrations of TRO on tube formation was particularly strong, suggesting that TRO may be acting at multiple levels to inhibit this complex process.

It is important to note that RPE cells were similarly inhibited by the PPAR-γ ligands in their response to VEGF. RPE cells are one of the few normal cell types, other than ECs, that express high-affinity VEGF-Rs and respond to VEGF as a mitogen and chemotactic agent. The RPE cells occupy a critical position at the interface between the neural retina and the choroid; therefore, inhibiting the uptake of VEGF by the RPE cells inhibits VEGF-induced angiogenesis.

Figure 8. TRO inhibits leakage of fluorescein dye in laser-induced CNV in the monkey. Seven photocoagulation burns were delivered in the paramacular region. Immediately after photocoagulation, two eyes received intravitreous injection of TRO (250 µM in DMSO, 100 µl), and two other eyes received only vehicle. CNV formation was detected as fluorescein leakage from the photocoagulation lesions in late phase (5.25 minutes) fluorescein angiography at 3 months. (A) Control animals show prominent fluorescein leakage from the laser spots, whereas in (B) TRO-treated animals showed a marked decrease in fluorescein leakage. Quantitation of these data is shown in Table 1.
choroid. In neovascular AMD they proliferate, migrate from the monolayer, and secrete VEGF, making them a central regulator of the neovascular process.3,39 Thus, inhibiting their growth can help to prevent a potentially initiating and amplifying event in the neovascularization process.

The molecular basis for the inhibition of CEC and RPE cell proliferation and migration and CEC tube formation is currently unclear. It is likely that PPAR-γ ligands act by inhibition of transcription factor function and that this effect is mediated by their central DNA-binding domain that recognizes response elements in promoters of specific target genes.10 We have previously shown that TRO inhibits the activity of ELK-1, an ets-family transcription factor, after mitogenic stimulation of vascular smooth muscle cells by bFGF. Similarly, PPAR-γ ligands regulate certain macrophage functions through transrepression of several transcription factors, including nuclear factor (NF)-xB and AP-1.21 Only one previous study has reported the effect of PPAR-γ ligands on angiogenesis in vivo. In that study of experimental corneal angiogenesis, hydron pellets containing VEGF with or without a PPAR-γ ligand were implanted in a corneal pocket and neovascular area measured after 6 days. 15d-PGJ2, a putative physiologic PPAR-γ ligand significantly inhibited VEGF-induced neovascular area in this model. Although such an assay provides support for the potential use of these ligands in modulating angiogenesis, a more stringent test of their efficacy is provided by use of an animal model in which angiogenesis is generated by naturally occurring wound healing responses. The laser photoagulation–induced CNV model, originally described in the monkey37 and more recently translated to the rat,38,44 induces a localized wound-healing response in the outer retina. Pathologic studies of these lesions reveal evidence of migration and proliferation of RPE cells, infiltration by macrophages, increased local expression of VEGF, and growth of new vessels into the subretinal space.35-40 These membranes have similar complex pathologic features in common with human CNV membranes,9 and they exhibit leakage of fluorescein dye, a dysfunction critically associated with active human CNV membranes.40 Our demonstration that intravitreal injection of TRO (days 1, 3, and 7) in rats dramatically inhibited both the percentage of lesions that show leakage as well as the amount of leakage per lesion makes a strong case for the functional value of this drug in the inhibition of pathologic neovascularization in the complex environment of the damaged outer retina. The functional study is supported by the histologic study demonstrating that the lesions were also thinner than those from untreated control eyes and contained reduced populations of RPE cells and CECs. The mechanism by which TRO inhibited CNV formation in this model is likely to be multifactorial. TRO inhibited VEGF-induced CEC and RPE cell migration and proliferation, which are critical features of the pathogenesis of CNV. In vivo this direct effect may be augmented by inhibition of VEGF-R expression; HUVECs treated with 15d-PGJ2 showed decreased expression of both VEGF-R1 and -R2.28 TRO may also inhibit extracellular matrix degradation, a process critical to neovascularization; PPAR-γ ligands are strong inhibitors of macrophage activation and have been shown to reduce matrix metalloproteinase (MMP)-9 activity in these cells.22 They also have been shown to inhibit proteolysis by inhibiting expression of urokinase plasminogen activator and stimulating expression of plasminogen activator inhibitor-1 in HUVECs.29 It is likely that a combination of effects resulting from inhibition of macrophage, RPE, and endothelial cell activation leads to the antiangiogenic response of PPAR-γ ligands.

The significant differences between the retinal structure of the rat and primates led us to assess the effects of PPAR-γ ligand treatment in a primate model. In particular, the macula, a region specialized for color and detailed vision in the primate is not found in the rat eye. In humans, CNV membranes in exudative AMD occur predominantly in the macular region; therefore, our primate studies were performed using paramacular laser photoagulation. No evidence of acute toxicity was found by electrophysiology (ERG) in either the rat or primate models. Electrophysiologic studies were not performed at later time points, and late functional abnormalities therefore cannot be ruled out; however, light microscopic histologic studies at day 14 in the rats and at 3 months in the primates showed no abnormalities in retinal regions away from the laser burns, including the macula in the primate eyes. Of interest, TRO treatment of laser-induced CNV lesions in the monkey resulted in a similar trend of inhibition of fluorescein leakage, although the number of animals required to demonstrate a statistical effect could not be studied. The apparent decrease in effectiveness of the drug in the monkey may have been a result of the single injection of TRO compared with three injections in the rat. The intraocular route of administration of TRO is practical for model systems, because the drug is localized to the site of disease; however, systemic administration is more appropriate for AMD patients.44 We have demonstrated the efficacy of PPAR-γ ligands in the inhibition of cells critically involved in the pathogenesis of CNV in vitro and the dramatic effect of one of these ligands (TRO) on CNV formation and function in vivo. Together, these studies implicate PPAR-γ as an important potential target for therapy in exudative AMD. The significance of this study may have widespread implication in other neovascular disorders.2

Acknowledgments

The authors thank Laurie LaBree for assistance in statistical analysis, Christine Spee for culture of endothelial cells, and Ernesto Barron for preparation of figures.

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