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Systemic rapamycin inhibits retinal and choroidal neovascularization in mice

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Purpose: Rapamycin exhibits significant antitumor/antiangiogenic activity that is coupled with a decrease in vascular endothelial growth factor (VEGF) production and a reduction in the response of vascular endothelial cells to stimulation by VEGF. VEGF plays a significant role in neovascular pathologies of the eye, thus we tested the possibility of using rapamycin to inhibit retinal and choroidal neovascularization (CNV).
Methods: CNV was induced in adult mice with laser photocoagulation. Retinal neovascularization was induced using the retinopathy of prematurity (ROP) hyperoxia/hypoxia model. Experimental animals received intraperitoneal (ip) injections of rapamycin (2 mg/kg/day or 4 mg/kg/day) for 1-2 weeks. Controls were not treated or received ip injections of phosphate buffered saline (PBS). Eyes were analyzed histologically for evidence of CNV or retinal neovascularization. ROP eyes were further analyzed for changes in VEGF and VEGF receptor (Flt-1 and Flk-1) protein content following rapamycin treatment.
Results: Rapamycin significantly reduced the extent of neovascularization in both the CNV and the ROP model. Immunohistochemical staining of treated and untreated ROP retina did not reveal a significant reduction in levels of VEGF protein or its receptors. Immunostaining for Flt-1 increased, while no obvious changes in Flk-1 were observed. Quantitative analysis of total protein via enzyme linked immunosorbent assay (ELISA) confirmed an increase in Flt-1 and VEGF, following drug treatment, with no effect on Flk-1.
Conclusions: These results suggest rapamycin may provide an effective new treatment for ocular neovascularization.

Age related macular degeneration (AMD) and retinopathy of prematurity (ROP) are characterized by aberrant ocular neovascularization that often results in severe, irreversible visual impairment. AMD is primarily responsible for vision loss in people over the age of 65 [1-3]. In the exudative (wet) form, choroidal blood vessels grow through Bruch's membrane into the subretinal space, resulting in accumulation of blood beneath the retina. As a result, retinal detachment and disciform scarring occur [4]. In contrast, ROP is characterized by retinal neovascularization that results from the exposure of the incompletely vascularized retina to high oxygen levels followed by a return to normoxic conditions, resulting in a relative hypoxic state [5]. This disease is a major cause of blindness in children in the developed world, and premature infants born less than 36 weeks gestation (<1250 g), requiring supplemental oxygen, are at greatest risk [6].

Mounting evidence strongly suggests that vascular endothelial growth factor (VEGF) plays a key role in these neovascular processes, and its inhibition can suppress the growth of abnormal blood vessels [7-12]. An upregulation of VEGF message in the ROP retina is well documented and its inhibition attenuates the observed retinal neovascularization [7-10]. In addition, VEGF has been associated with choroidal neovascular membranes, in RPE and maculae with AMD and in non-human primate and rodent laser models of choroidal neovascularization (CNV) [13-15]. Furthermore, subretinal injection of viral vectors encoding VEGF result in animal models of CNV [12,16,17].

Rapamycin, a macrolide fungicide with potent antimicrobial and immunosuppressive activities, possesses significant anti-tumor and anti-angiogenic properties [18,19]. The anti-angiogenic properties are associated with a decrease in VEGF production and a reduction in the response of vascular endothelial cells to stimulation by VEGF [19]. The mechanism by which rapamycin elicits its effects is not yet clear. Binding of rapamycin to its intracellular target, the immunophilin FKBP12, results in the inhibition of a kinase, termed the mammalian target of rapamycin (mTOR) [18]. mTOR, which is normally activated in response to growth signaling through the PI3K/Akt pathway, plays an important role in mitogenic cell signaling, affecting several biochemical processes that are critical in cell cycle regulation including translation initiation, DNA replication and protein synthesis [20]. Blockage of mTOR function inhibits PI3K/Akt signaling and results in cell cycle arrest [18].

In vitro studies in human smooth muscle and rodent endothelial cells indicate that mTOR inhibition by rapamycin specifically abrogates hypoxia triggered proliferation and angiogenesis [21]. Moreover, mTOR is an upstream activator of hypoxia inducible factor 1a (HIF-1a), a transcription factor that mediates the transcriptional regulation of VEGF [22,23]. In hypoxic cells rapamycin can interfere with HIF-1a activation by increasing the rate of its degradation [22]. Based on these findings, we tested the possibility of using rapamycin to

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inhibit neovascularization in a model of laser induced choroidal and ischemia induced retinal neovascularization, representing AMD [24] and ROP [25], respectively.

METHODS

Animals: All experiments were conducted in accordance with the Animal Care and Use Committee and The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Laser induced model of CNV: Adult female C57Bl/6 mice (n=27) were anesthetized with avertin (2,2,2-tribromoethanol; 0.5 mg/g intraperitoneally (ip)) and pupils were dilated with 1% tropicamide. Laser photocoagulation was performed using a diode laser photocoagulator (OcuLight Six; IRIS Medical, Mountain View, CA) and a slit lamp system with a cover slip as a contact lens. Laser photocoagulation (130 mW, 75 µm spot size, 0.1 s duration) was applied to the 9, 12, and 3 o'clock positions, 2 to 3 disk diameters from the optic nerve of each eye. The rupture of Bruch's membrane was identified by a bubble formation (which occurred immediately) at the site of photocoagulation. Laser spots that did not result in the rupture of Bruch's membrane were excluded from the studies. Two weeks post-treatment, the size of CNV was measured using a flat mount technique, as previously described [11]. Briefly, mice were anesthetized and perfused through the left ventricle with a 30 gauge needle with 1 ml of 50 mg/ml fluorescein labeled dextran ($2x10^6$ average molecular weight; Sigma, St. Louis, MO). The eyes were enucleated and fixed in 10% formalin for 24 h. Choroidal flat mounts were made and analyzed by fluorescent microscopy using a Leica DMR microscope (Leica Microsystems, Wetzler, Germany) equipped with epifluorescence illumination. Openlab software (Improvision, Boston MA) was used to measure the magnitude of the hyperfluorescent areas corresponding to choroidal neovascularization. In addition, some eyes were processed for histology. Eyes were enucleated, fixed in 4% paraformaldehyde overnight, cryopreserved and 10 µm sections were obtained on a Reichert-Jung Cryocut 1800 (Leica Microsystems). Sections were subsequently stained with hematoxylin and eosin.

Hypoxia induced model of ROP: We used a murine model of ROP that was described previously and in which retinal neovascularization is induced reproducibly 5-9 days after return to room air [25]. Sixty-five ROP mice were created for 4 different experiments. Briefly, post-natal day (P) 7 C57Bl/6 pups were placed in a hyperoxic chamber (75% oxygen) for 5 days. On P12, the mice were removed from the chamber and placed in room air. A subset of animals was sacrificed on day P19, and the eyes were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned serially (5 µm) and stained with hematoxylin and eosin. An individual masked to the treatment groups examined the sections for evidence of neovascularization via light microscopy. Neovascularization was quantified by counting the number of retinal vascular endothelial cell nuclei anterior to the inner limiting membrane (ILM). Counts were performed in transverse sections through the center of the eye (and the optic nerve head; see Figure 1D). Six to seventeen sections/eye were counted and counts were averaged.

In vivo rapamycin studies: Mice underwent laser photocoagulation as described above. Experimental groups received 4 mg/kg/day (n=9) or 2 mg/kg/day (n=9) of Rapamune (sirolimus; Wyeth Laboratories, Philadelphia, PA) ip starting the day of laser photocoagulation and then daily for 2 weeks. The control group (n=9) received PBS ip using the same schedule. The area of CNV was quantified as described above. ROP mice were removed from the hyperoxic chamber on P12. Animals subsequently received ip rapamycin injections for 7 days (2 mg/kg/day or 4 mg/kg/day). Pups were sacrificed at different time points. Eyes were enucleated and processed for paraffin (see above) or frozen and analyzed for levels of VEGF, Flt-1 and Flk-1 (see below). Control animals included: 1. age matched animals that developed normally in room air; 2. age matched animals that developed normally in room air and received rapamycin injections; 3. age matched animals that were exposed to hyperoxia and then returned to room air but did not receive rapamycin treatments. Experiments were repeated two times.

VEGF, Flt-1, and Flk-1 immunohistochemistry: Immunostaining was performed on frozen sections. Briefly, animals were euthanized and eyes were fixed in 4% paraformaldehyde. Following cryopreservation, 10 µm sections were obtained and blocked in PBS containing 1% horse serum, 1% BSA and 0.05% Triton X-100 in PBS for 40 min. Sections were subsequently treated with primary antibody directed against VEGF (VEGF A-20 rabbit polyclonal IgG, 1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Flt-1 (Flt-1 H-225 rabbit polyclonal IgG, 1:100 dilution; Santa Cruz Biotechnology, Inc.) or Flk-1 (A-3 mouse monoclonal IgG, 1:50 dilution; Santa Cruz Biotechnology, Inc.) prepared in PBS containing 1% horse serum, 1% BSA and 0.3% Triton X-100 (1 h room temp). Sections were washed with PBS and then incubated in secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (H+L), 1:100 dilution, Molecular Probes, Eugene, OR or Alexa Fluor 488 goat anti-mouse IgG (H+L), 1:100 dilution, Molecular Probes) prepared in PBS containing 1% horse serum, 1% BSA and 0.3% Triton X-100 (1 h at ambient room temperature). Slides were washed with PBS, coverslipped with mounting media containing DAPI and evaluated with a Leica DME microscope (Leica Microsystems, Inc.) equipped with epifluorescence. Images were captured with a Hamamatsu digital camera and Openlab 2.2 image analysis software (Improvision, Inc.). To test the specificity of antibody, control sections were stained simultaneously following the same procedures; however, primary antibody was omitted. Alternatively, an irrelevant primary antibody was added.

VEGF, Flt-1, and Flk-1 enzyme linked immunosorbent assays: Upon enucleation, conjunctiva was removed, and eyes were stored at -80 °C. Samples were homogenized in a buffer containing 10 mM KH_2PO_4, 150 mM NaCl, 200 mM sucrose, 10 mM EDTA, 10 mg/ml leupeptin, 10 mg/ml pepstatin A and 1 mM PMSF. Samples were subsequently centrifuged at 6,500 rpm for 7 min. The supernatants were analyzed for protein concentrations via the Bio-Rad Protein Assay Reagent (Bio-

Rad Laboratories, Hercules, CA). Samples were diluted to a final concentration of 2 mg/ml. Levels of VEGF, Flt-1, and Flk-1 were determined using the Quantikine M Murine VEGF, sVEGF R1, and sVEGF R2 Immunoassay Kits, respectively (R&D Systems Inc., Minneapolis, MN). Five to 14 tissue samples were analyzed for each group and time point.

Statistical analysis: The statistical significance of differential findings between experimental and control groups was determined by an unpaired Student's t-test and considered significant if two tailed p values were <0.05.

RESULTS

Effect of rapamycin administration on CNV: Animals that received rapamycin ip for 2 weeks did not manifest any significant side effects such as weight loss, severe infection, or death due to the drug, and maintained normal appetite and activity. The laser induced model yielded a mean area of CNV equal to 0.055±0.006 (SEM) mm^2 in the control (no rapamycin) group, 0.039±0.004 mm^2 in the 2 mg/kg group, and 0.033±0.003 mm^2 in the 4 mg/kg rapamycin group, as indicated by analysis of choroidal flat mounts (Figure 2A,B,E).

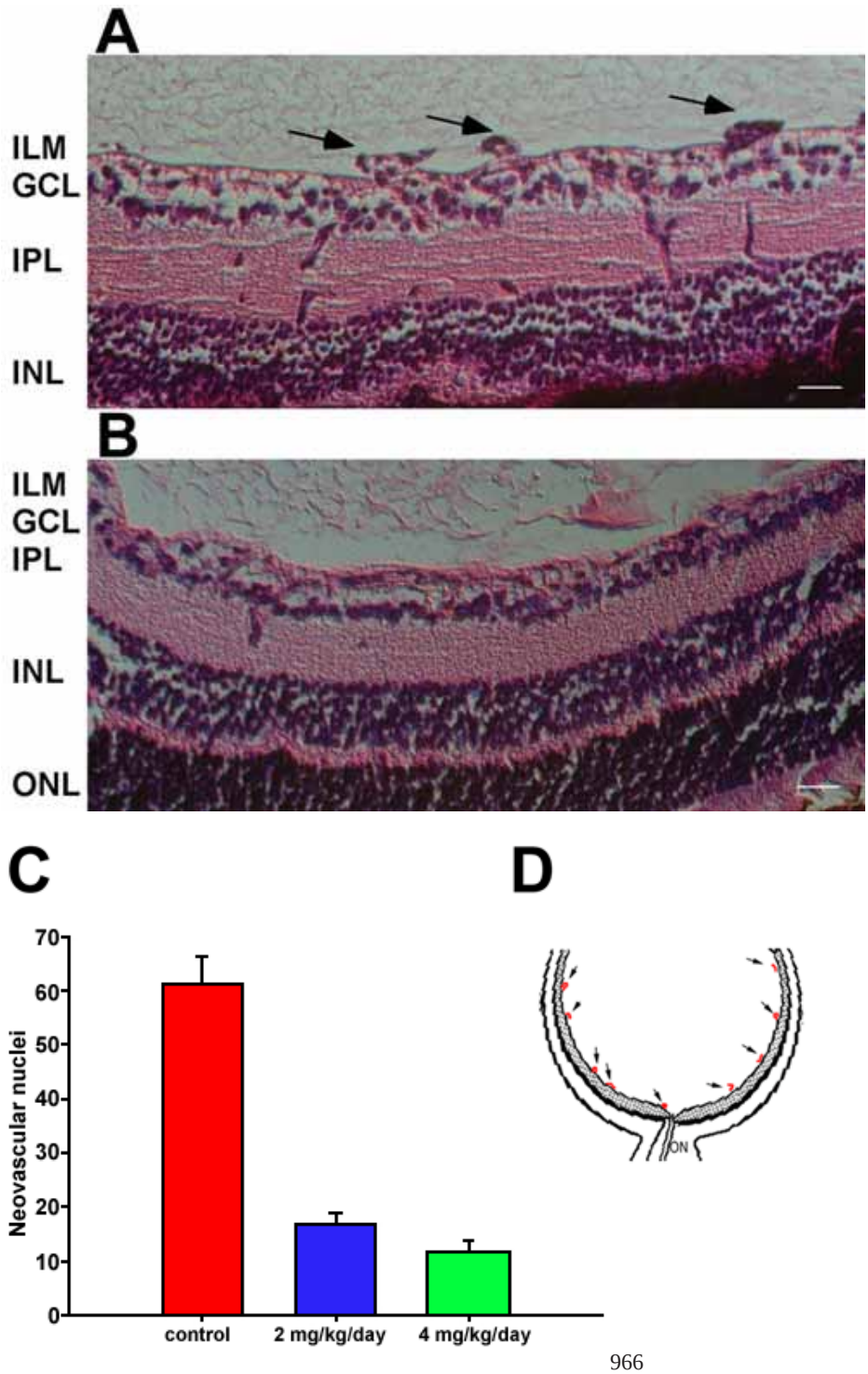

Figure 1. Rapamycin significantly reduces neovascular lesions crossing the ILM in ROP mice. **A**: Representative section (20x) of a control P19 ROP retina. **B**: Representative section (20x) from a P19 ROP animal treated with 2 mg/kg rapamycin. Arrows highlight neovascular tufts protruding into the vitreous. Scale bars in **A** and **B** represent 40 μm. **C**: The average number of vascular nuclei found anterior to the ILM per 5 μm cross section were determined for each experimental group. The error bars represent (SEM). **D**: Cartoon indicating location of pathologic neovascular nuclei. Arrow, neovascular fronds; ON, optic nerve.

This translated into a 29.8% decrease in CNV area in the low dose group and a 40% decrease in the high dose group, with respect to the control, that was statistically significant ($p<0.05$ and <0.001, respectively). Representative cryopreserved histological sections show the difference in area of neovascularization before and after treatment (Figure 2C,D).

Effect of rapamycin administration on retinal neovascularization: Mice with oxygen induced ischemic retinopathy received ip injections of rapamycin (2 mg/kg or 4 mg/kg) once daily between P12 and P18. Age matched normoxic animals also received drug treatment. Additional normoxic and ROP controls were maintained without thera-

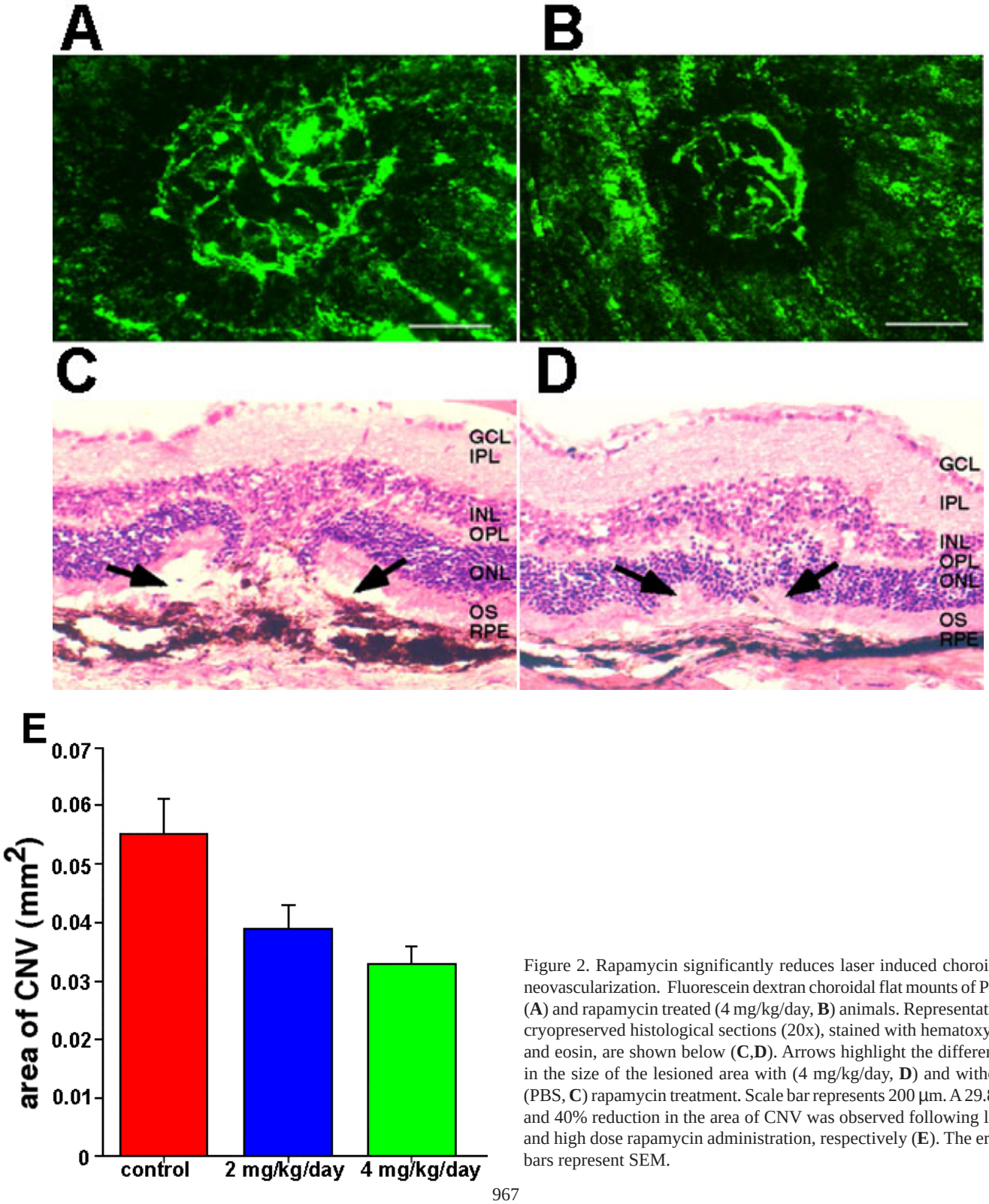

Figure 2. Rapamycin significantly reduces laser induced choroidal neovascularization. Fluorescein dextran choroidal flat mounts of PBS (**A**) and rapamycin treated (4 mg/kg/day, **B**) animals. Representative cryopreserved histological sections (20x), stained with hematoxylin and eosin, are shown below (**C**,**D**). Arrows highlight the difference in the size of the lesioned area with (4 mg/kg/day, **D**) and without (PBS, **C**) rapamycin treatment. Scale bar represents 200 µm. A 29.8% and 40% reduction in the area of CNV was observed following low and high dose rapamycin administration, respectively (**E**). The error bars represent SEM.

peutic intervention. Rapamycin had no apparent systemic effects on the normoxic animals. Hypoxic animals exhibited weight loss for 3-4 days of treatment, but then slowly regained weight. The ROP model yielded 61.3±5.0 (SEM) vascular nuclei per 5 μm cross section, extending from the ILM into the vitreous on P19. This number was significantly reduced following daily rapamycin treatments (Figure 1). The 2 and 4 mg/kg groups averaged 16.9±2 ($p<0.001$) and 11.6±2 ($p<0.001$) nuclei, respectively. This data correlated with a 72.4% decrease in neovascular tuft extension into the vitreous with low dose rapamycin and an 81.1% reduction with the high dose.

VEGF, Flt-1, and Flk-1 immunohistochemistry: Immunohistochemistry was performed in order to assess whether

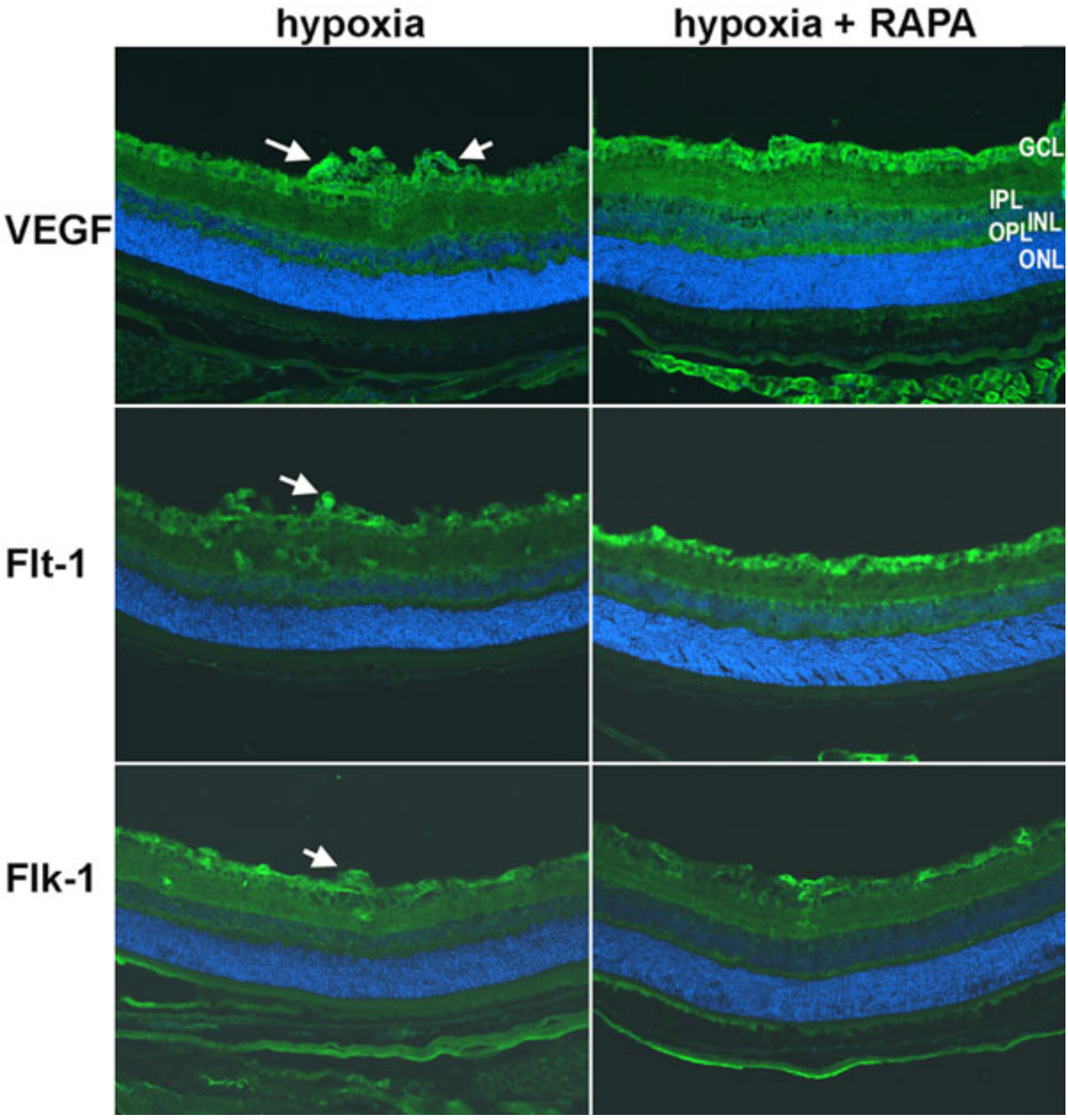

Figure 3. VEGF, Flt-1, and Flk-1 immunostaining in the ROP retina on P19. Magnification of all images are 10x. VEGF, Flt-1, and Flk-1 positive cells are stained in green; Nuclei are labeled blue with DAPI. VEGF protein is localized to cells throughout the eye with the strongest signal in the ganglion cell layer (GCL), surrounding the neovascular tufts (arrows). Rapamycin treatment (2 mg/kg/day or 4 mg/kg/day) has no obvious effect on the overall staining pattern of VEGF in the retina. Flt-1 and Flk-1 are also primarily localized inner plexiform layer (IPL), outer plexiform layer (OPL) and GCL. An increase in Flt-1 is observed with low and high dose rapamycin treatment in the GCL. INL represents inner nuclear layer. Scale bars represent 20 μm.

rapamycin treatment affected patterns of distribution of VEGF protein and its receptors in the ROP retina (Figure 3). We observed VEGF protein throughout the retina, with highest levels in the ganglion cell layer (GCL), especially surrounding the neovascular tufts. VEGF levels were also high in the outer plexiform layer (OPL), inner plexiform layer (IPL). The two receptors showed expression in these regions. Similar patterns of expression were observed in the age matched normoxic controls (data not shown). Interestingly, high dose rapamycin increased Flt-1 labeling. No obvious changes were readily detectable in VEGF or Flk-1 labeling. Similar patterns of expression were seen with the low dose group (data not shown).

Quantitative analysis of VEGF, Flt-1, and Flk-1: In order to confirm our immunohistochemical observations, we used enzyme linked immunosorbent assays (ELISAs) to quantify VEGF, Flt-1 and Flk-1 levels in the eye. Conjunctiva was removed from all samples at the time of enucleation, as VEGF protein is normally found in this tissue in rodents and non-human primates [26]. Our data indicate rapamycin does not reduce levels of VEGF or its receptors in either the normal or ROP developing eye (Figure 4). Upon initial removal of animals from the hyperoxia chamber, VEGF protein levels were similar to those of untreated 12 day old controls (35.2±12.4 pg/ml and 43.4±3.6 pg/ml, respectively). However, VEGF concentrations in the treated mice increased significantly by day 15 (174.7±7.6 pg/ml) and remained elevated (Figure 4A). Rapamycin treatment resulted in comparably high VEGF levels on postnatal (P) day 15 (181.1±12.9 pg/ml), and these levels remained high through all the time points examined. No effect was seen on VEGF protein levels in eyes of animals raised in room air, where levels remain low and slowly decrease over time.

Flt-1 levels in ROP mice were also analogous to those of untreated controls on P12 (1762.5±155.4 pg/ml compared to 1695.6±175.9 pg/ml; Figure 4B). A gradual rise in receptor content is observed in the ROP group between P13-P17, but by P19 levels begin to decline. Rapamycin treatment increased Flt-1 levels in the ROP group and had no effect on the controls. Interestingly, we observed a significant elevation in Flk-1 following oxygen exposure (from 35.0±4.9 pg/ml to 11.8±5.4 pg/ml in age matched normoxic controls) that decreases to normal levels by P15 (16.2±1.8 pg/ml; Figure 4C). Rapamycin had no apparent effect on the decrease we observed, nor did it affect overall levels in developing eyes of animals maintained under normoxic conditions.

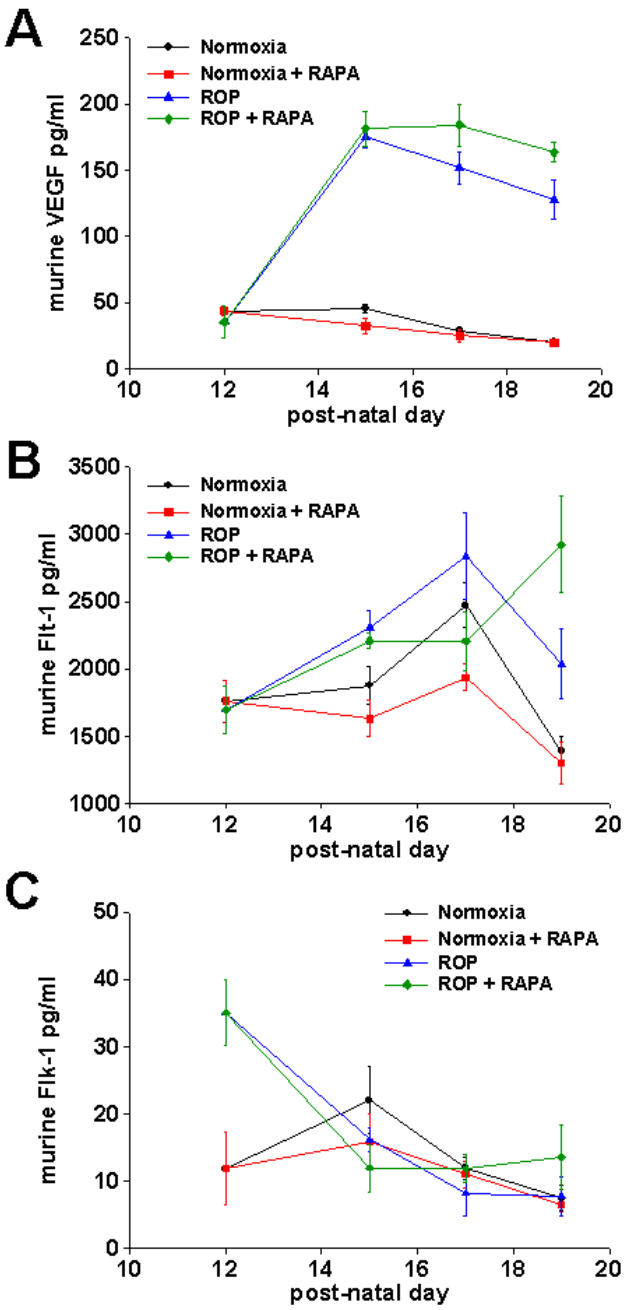

Figure 4. VEGF, Flt-1, and Flk-1 protein levels in developing normal and ROP eye were quantified using ELISA. **A**: Upon removal of mice from the hyperoxia chamber, VEGF levels increase dramatically. ROP animals continue to maintain high levels of VEGF with rapamycin treatment (4 mg/kg/day). By P19, the level of VEGF in ROP animals receiving drug is higher than that of ROP controls (163.6±7.2 pg/ml compared to 127.8±14.4 pg/ml). **B**: A general increase in Flt-1 is observed in normoxic and ROP animals from P12-P17, followed by a decrease from P17-P19. High dose rapamycin had no effect on overall levels of Flt-1 in normoxic animals at any time point. In contrast, ROP animals treated with drug exhibit an overall increase in Flt-1 from P17-P19 that is not observed in any other group. **C**: Hyperoxia causes an increase in Flk-1 in the eye (compare 35.0±4.9 pg/ml [ROP] to 11.8±5.4 pg/ml [normoxia] at P12). By P15 Flk-1 levels in ROP animals drop to those of normoxic controls. Rapamycin (4 mg/kg/day) has no effect on ROP or normoxic animals at any time point examined.

DISCUSSION

In the present study, we demonstrated the efficacy of ip rapamycin for the treatment of CNV and retinal neovascularization in mice. Both pathologies have been associated with increased levels of VEGF [5-7,13-15]. Thus, we examined the effects of rapamycin on levels of VEGF and its receptors in the ROP retina. Studies in a murine tumor model demonstrated that administration of rapamycin reduced VEGF production and decreased the vascular endothelial cells' sensitivity to VEGF stimulation [19]. The murine *VEGF* gene is alternatively transcribed, resulting in three different isoforms

($VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$) that differ in molecular mass, solubility, receptor binding, and function [27]. While we are not aware of any reports that specifically link rapamycin's effect to any one single isoform, it is known that during pathological ischemia induced retinal neovascularization, absolute and relative expression levels of $VEGF_{164}$ are increased, and it selectively induces inflammation and cellular immunity [28]. In addition, it has been identified as an important isoform in the pathogenesis of early diabetic retinopathy [29].

In vitro studies revealed rapamycin is capable of blocking the activation of HIF-1a, the transcription factor that induces the expression of VEGF and its receptor Flt-1 [22,23]. Curiously, our ELISA and immunohistochemical data revealed that VEGF levels are not reduced in the ROP model. Instead, these levels increase following treatment even though the extent of neovascularization is significantly reduced. Interestingly, Flt-1 protein levels also increase following treatment, suggesting that Flt-1 may play a role in minimizing the pathological changes in the ROP retina. In contrast, we did not observe any changes in Flk-1 levels after treatment with rapamycin.

It is not apparent by what mechanism rapamycin could increase Flt-1. Flt-1 has been localized to neonatal blood vessels, while Flk-1 is selectively localized outside the vasculature in the neonatal retina until P12, when it is also seen on retinal vessels [30]. While Flt-1 message is upregulated between P3 and P26, there are no significant changes in Flk-1 mRNA expression during this time. The individual involvement of Flt-1 and Flk-1 in oxygen induced retinal vessel degeneration has been examined in vivo using specific ligands that selectively bind each of the receptors [30]. Specific activation of Flt-1 with placental growth factor-1 (PlGF-1) protects against oxygen induced vessel loss without stimulating vascular proliferation and neovascularization; however, VEGF-E, which selectively activates Flk-1, does not prevent oxygen induced vaso-obliteration. These findings strongly suggest that stimulating Flt-1 by PlGF-1 may prove to be an attractive strategy for preventing ROP. Transforming growth factor b1 (TGF-b1) also induces Flt-1 expression in endothelial cells, and co administration of TGF-b1 and PlGF-1 further increase protection from hyperoxia induced degeneration [31].

It has been suggested that Flt-1 can act as a "decoy" receptor to prevent the binding of VEGF to Flk-1, the major mediator of the mitogenic, angiogenic and permeability enhancing effects of VEGF [32]. Studies with a soluble form of Flt-1 show it causes both the sequestration of VEGF and forms inactive heterodimers with membrane spanning isoforms of Flt-1 and Flk-1 [33,34]. Indeed, it has been demonstrated that viral mediated delivery of a soluble form of Flt-1 results in an inhibition of corneal, choroidal and retinal neovascularization [32,35-37].

Rapamycin may also be exerting its effects through additional non-VEGF related pathways. One possibility is that rapamycin is blocking the production of other powerful pro-angiogenic agents, as high levels of VEGF are not sufficient to induce the neovascularization observed in ROP and CNV, and VEGF inhibitors only partially block the neovascular response [38,39]. Hypoxia can stimulate gene expression by at least three different mechanisms including gene transcription, regulation at the translational level and mRNA stabilization, and mTOR, the target of rapamycin, has effects on all of these pathways [18-20,40]. Factors such as basic fibroblast growth factor (bFGF), platelet derived growth factor B (PDGF-B), nitric oxide synthase and angiopoietin are upregulated by hypoxia [21]. In a study that examined the effects of VEGF, bFGF, and PDGF-B on reconstituted basement membrane matrix experiments, it was found that only bFGF and PDGF-B could exert a secondary sprouting effect alone [41]. Alternatively, rapamycin may be directly or indirectly stimulating the production of one or more anti-angiogenic factors that are responsible for the dramatic effects we observed. Anti-angiogenic molecules such as pigment epithelium derived factor (PEDF), tissue inhibitor of metalloproteinase-3 (TIMP3) and endostatin have all shown efficacy in inhibiting both CNV and retinal neovascularization [42].

The mechanism by which rapamycin elicits its anti-angiogenic properties in the eye is not clear. Nevertheless, this study provides evidence that rapamycin inhibits ocular neovascularization in two different models. These results warrant further testing to determine the stability of the effect and the effective dose range of the drug. We selected our dosages based on a previous report that indicated 1.5 mg/kg/day rapamycin (about 40 ng/ml blood trough level) was effective in inhibiting tumor angiogenisis and decreasing VEGF production [19]. Adult transplant patients on a rapamycin regimen maintain a dose of 2 mg/day following an initial one-time dose of 6 mg [43], with recommended blood trough levels of 4-20 ng/ml [44]. The antiangiogenic effects we observed did not significantly differ between the two concentrations of drug we tested, and it may be possible to achieve similar effects with substantially lower doses. It may also be possible to deliver rapamycin locally to the eye via topical or subretinal injection in order to limit side effects that might appear after systemic exposure. These possibilities and the relative safety of this approach, with respect to the eye and the rest of the body, are under investigation.

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