

Inhibition of VEGF Secretion and Experimental Choroidal Neovascularization by Picropodophyllin (PPP), an Inhibitor of the Insulin-like Growth Factor-1 Receptor

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INTRODUCTION. Choroidal neovascularization (CNV) is a debilitating complication of age-related macular degeneration (AMD) and a leading cause of vision loss. Along with other angiogenic factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-1 and its receptor, IGF-1R, have been implicated in CNV.

PURPOSE. A prior study has shown that the cyclolignan picropodophyllin (PPP) efficiently blocks the insulin-like growth factor-1 receptor (IGF-1R) activity and causes cell death in uveal melanoma cell lines and in an in vivo model. In this study we investigated the effect of PPP on VEGF expression, both in vitro and in vivo, and whether this effect has antiangiogenic consequences in a murine CNV model.

METHODS. C57BL/6J mice with laser-induced CNVs were treated with PPP. Effects on CNV area were assayed by image analysis. VEGF levels in the choroid and retinal pigment epithelial cells (ARPE-19) were measured by Western blot or ELISA. Transcriptional activation of the VEGF promoter was determined by luciferase reporter gene assay.

RESULTS. Mice treated with PPP, administered intraperitoneally or orally, showed a 22% to 32% ($P = 0.002$) decrease in CNV area. Furthermore, VEGF levels in the choroid were significantly reduced. In cultured ARPE-19 cells, IGF-1 was shown to increase VEGF secretion. This increase was completely blocked by PPP. PPP reduced the level of transcriptional activity of the VEGF promoter.

CONCLUSIONS. PPP reduces IGF-1-dependent VEGF expression and CNV in vivo. Accordingly, IGF-1R inhibitors may be useful tools in the treatment of conditions associated with CNV,

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Neovascular age-related macular degeneration (AMD) associated with choroidal neovascularization (CNV), the proliferation of blood vessels into the subretinal space from the underlying choriocapillaris, is the leading cause of blindness among the elderly in industrialized countries.^{1,2}

Studies of choroidal neovascular membranes from patients with AMD have demonstrated the presence of various angiogenic and growth factors, including vascular endothelial growth factor (VEGF),^{3,4} transforming growth factor (TGF)- β ,⁵ and insulin-like growth factor 1 (IGF-1).⁶ VEGF is a major angiogenic stimulant in the development of CNV^{7,8} and also modulates vascular permeability. VEGF is produced by several cell types during the pathogenesis of CNV, including infiltrating macrophages.^{9,10} Another important source of VEGF is likely to be the retinal pigment epithelial (RPE) cells, which are known to produce VEGF.^{7,11} It is not clear, however, how RPE cells are stimulated to release VEGF in the chain of events leading to CNV. A potential role of IGF-1 and -1R in the pathogenesis of CNV has been discussed but still has not been confirmed. The source of IGF-1 is thought to be neurons, since these have been shown to secrete IGF-1 under metabolic stress.¹² RPE cells represent another IGF-1 source,¹³ and IGF-1 has been shown to stimulate secretion of VEGF in RPE cells.¹³

We have shown that picropodophyllin (PPP), a small molecule belonging to the cyclolignan family, inhibits phosphorylation of IGF-1R, blocks its downstream signaling, and causes tumor regression in vivo.^{14–22} A recent study showed that PPP prolongs survival drastically in a model of multiple myeloma.²³ The animals were treated daily up to 150 days, and survival was prolonged to 3 months compared with the control group.²³ This result suggests that PPP-induced IGF-1R inhibition not only exerts a high antitumor efficacy but also is well tolerated in vivo. Recently, we demonstrated that PPP blocks uveal melanoma growth and metastases in vivo.²⁴

To elucidate the potential therapeutic role of IGF-1R inhibition in neovascular AMD, we undertook this study to explore the effects of PPP on VEGF secretion and experimental CNV in a mouse model. Our data demonstrated that PPP inhibited IGF-1 induced VEGF secretion in RPE cells, and it reduced VEGF protein expression and CNV formation in vivo.

MATERIALS AND METHODS

Reagents

PPP was synthesized as described¹⁴ and was dissolved in DMSO (0.5 mM) before addition to cell cultures, whereas for intraperitoneal (IP) injection of the mice, the compound was dissolved in a 10- μ L volume of DMSO+vegetable oil (10:1; vol/vol). The PPP/food mixture was prepared as follows: One hundred eighty milligrams PPP was first dissolved in 50 mL acetone. The solution was then added to a mixture

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of 500 g of food powder (Lactamin, Kimstad, Sweden) and acetone+sterile water (1:1) to make the food semiliquid. The final solution was then mixed thoroughly and dried overnight at 40°C. Mice eat approximately 5 g of food a day. The polyclonal antibody to the β subunit of the IGF-1R was from Cell Signaling Technology (Danvers, MA), whereas polyclonal antibodies to IGF-1(H-70), to VEGF (VG-1) and to GAPDH (FL-335) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animals

Male C57BL/6 mice between 6 and 10 weeks old were purchased from Charles River (Sulzfeld, Germany). The animals were given food and water ad libitum, and were maintained in pathogen-free, 12-hour light-dark conditions. For all procedures, anesthesia was achieved by subcutaneous injections of ketamine (10%; ratiopharm, Ulm, Germany)/xylazine (2%, ratio 16:1; Bayer, Leverkusen, Germany) and the pupils were dilated with phenylephrine HCl (0.25%)–tropicamide (0.05%). All animal experiments followed the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee of Karolinska Institute.

Murine Model of Laser-Induced CNV

To induce CNV, we induced ruptures of Bruch's membrane by krypton laser, as described previously.²⁵ Laser photocoagulation (110 mW, 100 ms, 50 μ m; Argon, Coherent Novus 2000; Carl Zeiss Meditec, Oberkochen, Germany) was performed in the right eye of each animal on day 0. Each three laser spots were applied in a standardized fashion around the optic nerve with a slit lamp delivery system and a coverslip used as a contact lens. Formation of evaporation blebs at the site of laser exposure indicated the effective rupture of Bruch's membrane, and only burns in which a bubble was produced were included in the study.

Pharmacologic Treatment of Mice with CNV

Three different experiments were performed with pharmacologic treatment of PPP. In the first experiment, C57BL/6J mice were divided into two treatment groups: group 1 received 20 mg/kg/12 h intraperitoneal injections of PPP in 10 μ L volume of DMSO+vegetable oil (10:1; vol/vol), and group 2 (control group) was treated with vehicle only. Twelve animals were treated in each group for a 2-week period. In the second experiment, each of the mice belonging to the treatment group were treated with a 5-mg PPP-food mixture per day for a 2-week period, and the mice in the control group were treated with 5 mg of normal food per day. In the third experiment, there were three different groups: Group 1 was composed of mice that received six laser spots around the optic nerve as described herein and then were treated for 2 weeks with 5 mg PPP-food mixture per day; group 2 included five mice that received six laser spots around the optic nerve and then were treated for 2 weeks with 5 mg of normal food per day; and group 3 consisted of five mice that had not received any laser spots and where fed with normal food.

Labeling and Quantification of CNV

The eyes were enucleated at the indicated times after krypton laser application and PPP or vehicle treatment and fixed in 4% paraformaldehyde for 30 minutes. The cornea and lens were removed, and then the entire retina was carefully removed. The RPE-choroid-sclera eyecups were rinsed in PBS, permeabilized in 0.1% Triton X-100, and blocked with 1% goat serum in PBS/Triton X-100. The eyecups were then incubated with 0.5% FITC isolectin B4 (lectin from *Griffonia simplicifolia*; Sigma-Aldrich, St. Louis, MO) overnight at 4°C, and the eyecups were flattened and mounted with antifade medium (Vectashield; Vector Laboratories, Burlingame, CA) with the sclera facing down and the choroid facing up. Flatmounts were examined with a fluorescence microscope (Axioskop 2; Carl Zeiss Meditec), and images were captured with a digital camera (Carl Zeiss Meditec) and further analyzed using the AxioVision LE software (Carl Zeiss Meditec). Each

lesion was manually measured in a masked fashion, and the total area of hyperfluorescence associated with each burn was recorded. The average CNV lesion area (in square micrometers) of each treated eye was treated as a single statistical point.

Histomorphology of PPP-Treated and Control CNV Lesions

In a separate experiment, we treated two groups of C57BL/6J mice, in which we induced experimental CNV in the right eye, as described earlier, with either 20 mg/kg/12 h IP of PPP or vehicle for 2 weeks. At the end of the experiment, the mice were killed, and the eyes were enucleated.

Formalin-fixed eyes were embedded in paraffin and serial sections (4 μ m thick) were cut throughout the entire extent of each laser burn and stained with hematoxylin-eosin. To evaluate the effect of PPP on CNV membranes, we examined hematoxylin-eosin-stained serial sections at 200 \times magnification with a light microscope (Axioscope; Carl Zeiss Meditec) and a digital color camera (Axiomat; Carl Zeiss Meditec), as previously described.²⁶

Cell Culture and VEGF Protein Detection with ELISA

Experiments were performed with ARPE-19 cells, a nontransformed human diploid RPE cell line that displays many differentiated properties typical of RPE in vivo.²⁷ ARPE-19 cells were plated at subconfluence and maintained in culture at 37°C in 5% CO₂. All ARPE-19 cultures were maintained at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM)+nutrient mixture F12+10% fetal bovine serum.

Culture medium was changed to 1% FBS 12 hours before treatment. The cells were treated at subconfluence for 25 hours with PPP and/or IGF-1 (100 ng/mL) for 24 hours. Conditioned medium was collected and the VEGF ELISA was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN), except that the conditioned medium was not diluted. The amount of VEGF protein was correlated to total protein determined with the Bradford reagent (Sigma-Aldrich).

Western Blot Analysis in the Choroidal and RPE Layers

IGF-1R, IGF-1, and VEGF protein expression was semiquantitatively evaluated by Western blot analysis of the choroidal and RPE layers from C57BL/6J mice divided into three groups: (1) mice that had no krypton laser applied (no laser group), (2) mice that had krypton laser applied and were treated with normal food (laser group), and (3) mice that had krypton laser applied and were treated with 5 mg PPP-food mixture per day (PPP+laser group). The treatment period was 2 weeks. Briefly, the vitreous and retina were removed, and the choroid and RPE layer were lysed for 30 min on ice in buffer (1%NP-40, 0.5% deoxycholate, 1% SDS, 150 mM NaCl, 50 mM Tris-HCl [pH 8]) supplemented with a mixture of protease inhibitors (Sigma-Aldrich). The samples were cleared by centrifugation (14,000 rpm, 30 minutes, 4°C) and assessed for protein concentration (Bradford assay; Bio-Rad Laboratories, Munich, Germany). Twenty micrograms of protein per sample were electrophoresed in a 10% Tris-glycine gel (Novex; Invitrogen, Carlsbad, CA).

After electrophoresis, the proteins were transferred overnight to nitrocellulose membranes (GE Healthcare, Uppsala, Sweden) and then blocked for 1 hour at room temperature in a solution of 5% (wt/vol) skimmed milk powder and 0.02% (wt/vol) Tween 20 in PBS (pH 7.5). Incubation with the appropriate primary antibodies was performed for 1 hour at room temperature (VEGF and GAPDH) or overnight (IGF-1R) at 4°C and was followed by washes with PBS and incubation with either an HRP-labeled or a biotinylated secondary antibody (GE Healthcare) for 1 hour. After the biotinylated secondary antibody, incubation with streptavidin-labeled horseradish peroxidase was performed. The detection was made with chemiluminescence (either ECL, GE Health-

care; or Supersignal West Pico; Pierce Biotechnology, Rockford, IL). The films were then scanned (Fluor-S; Bio-Rad).

IGF-1R, IGF-1, and VEGF protein expression levels from choroidal-RPE samples from C57BL/6J mice of all three groups were normalized to the corresponding expression of GAPDH control protein and expressed as arbitrary units. Three independent experiments were evaluated.

Transfection with VEGF Promoter-Driven Luciferase Construct

The construct was a kind gift from Dieter Manne (Institute of Molecular Oncology, Tumor Biology Center, Freiburg, Germany), and its use is described elsewhere.²⁸ In brief, the cells were cultured in a 96-well fast-transfection plate (Kinovate Life Sciences, Oceanside, CA) and transfected with 0.5 μ g of DNA/10,000 cells per well, in a serum-free medium, according to the manufacturer's instructions. After 24 hours, the cells were stimulated with 100 ng/mL IGF-1, in the presence or absence of 1 μ M PPP for another 24 hours. To enable the levels of transcripts to be quantified, standard curves were generated using serial dilutions of ARPE-19 cells. Negative controls consisting of cells transfected with empty vector were included. All transfections were set up in triplicate. The firefly luciferase activities was measured using a luciferase assay system kit (Promega, Madison, WI) and correlated to cell number using an XTT cell survival assay (Roche Diagnostics, Mannheim, Germany), as previously described.²⁹

Statistical Analysis

All results are expressed as the mean \pm SD. The results were compared by using analysis of variance (ANOVA) and Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of PPP on Laser-Induced CNV

After CNV induction with a krypton laser, PPP or vehicle (control) was administered either by IP or orally for 2 weeks. Each group consisted of 10 to 12 mice. We analyzed CNV lesion sizes on choroidal flatmount preparations. Both delivery of PPP IP (Fig. 1A) or orally (Fig. 1B) significantly inhibited the development of CNV compared with the control. Accordingly, measurements of all samples demonstrate that the sizes of the laser-induced CNVs in mice treated with intraperitoneal PPP was reduced by 22% ($P = 0.002$; Fig. 1A). In mice treated with PPP orally, the CNV sizes were reduced by 31.5% ($P = 0.002$; Fig. 1B).

Figures 2A–D show fluorescence microscopy of choroidal flatmounts after immunostaining with isolectin B4 to identify vessels. A representative flatmount from DMSO-treated mice (control) showed a large area of CNV (Fig. 2A). The CNV area from a PPP-treated mouse was smaller (Fig. 2B). Corresponding effects were seen in orally treated mice (Figs. 2C, 2D).

Morphology of PPP-Treated CNV Lesions

Histologic examination of CNV membranes after 14-day treatments (PPP or control) showed a domelike CNV complex emerging through the ruptured Bruch's membrane containing fibrovascular tissue with vascular endothelial cells and scattered RPE cells. No apparent damage to the neural retina was seen overlying the CNV complex (Figs. 2E–H). In the case of PPP treatment (Figs. 2F, 2H) a marked thinning of the CNV complex was observed compared to the control subjects (Figs. 2E, 2G). No statistical analysis was performed, since this method was considered less accurate compared with the flatmount technique.³⁰ Only representative pictures were taken from different mice of both groups, to show the structural

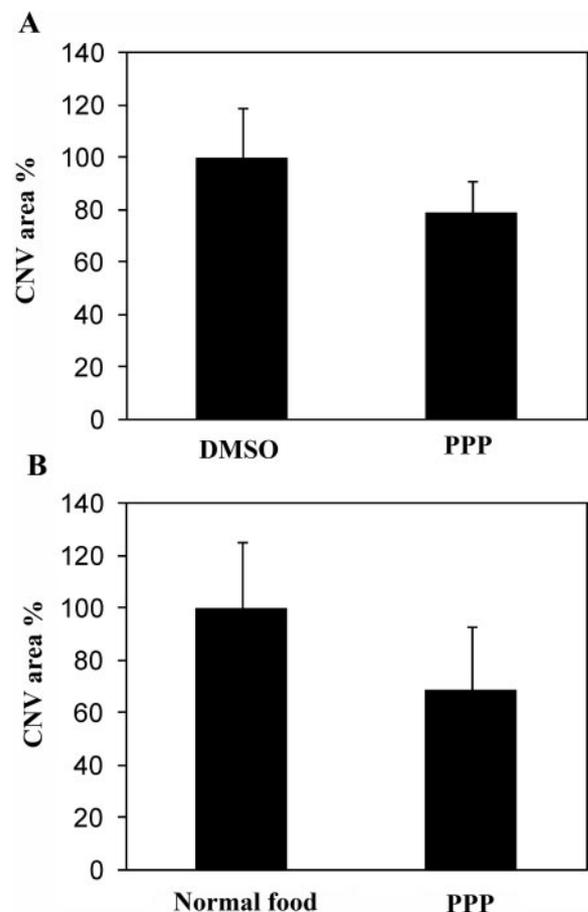


FIGURE 1. Effect of PPP on CNV in vivo. (A) CNVs were induced by laser. The mice were treated daily with DMSO (control) or PPP (20 mg/kg/12 h) by IP injection for 14 days. Each group involved 12 mice. CNV lesion sizes were measured by computerized image analysis. Control (DMSO) was considered to be a 100% CNV area. PPP reduced CNV lesion sizes significantly compared with control untreated animals ($P = 0.002$). (B) The mice were treated daily with control food or PPP-containing food (5 mg PPP-food mixture per day) for 14 days. Each group involved 12 mice. Quantification of CNV lesion sizes was performed. Oral PPP significantly reduced CNV sizes compared with control untreated animals ($P = 0.002$).

difference of the choroids and of the CNV in particular, after PPP treatment.

VEGF Secretion in RPE Cells

The effect of IGF-1 on VEGF secretion by ARPE-19 cells was measured by ELISA of the levels of VEGF in the medium. Serum-starved ARPE-19 cells were stimulated with IGF-1 for 24 hours. As shown, VEGF secretion was increased by 25% to 30% by IGF-1 (Fig. 3A). However, if the cells were co-incubated with PPP (1 μ M), the VEGF levels were almost normalized ($P < 0.05$). These suppressive effects of PPP on IGF-1-induced VEGF expression are consistent with those obtained in multiple myeloma¹⁷ and Kaposi's sarcoma³¹ cells.

We also investigated whether PPP affects transcriptional activity of the VEGF promoter in RPE cells. Hence, we made use of a VEGF luciferase reporter gene construct. As shown, untreated RPE cells (control) exhibited a background luciferase activity (Fig. 3B). On treatment with IGF-1, the luciferase activity increased 2.5-fold, but when PPP (1 μ M) was incubated along with IGF-1, the luciferase activity decreased by 25% ($P = 0.000009$), as related to the control (Fig. 3B).

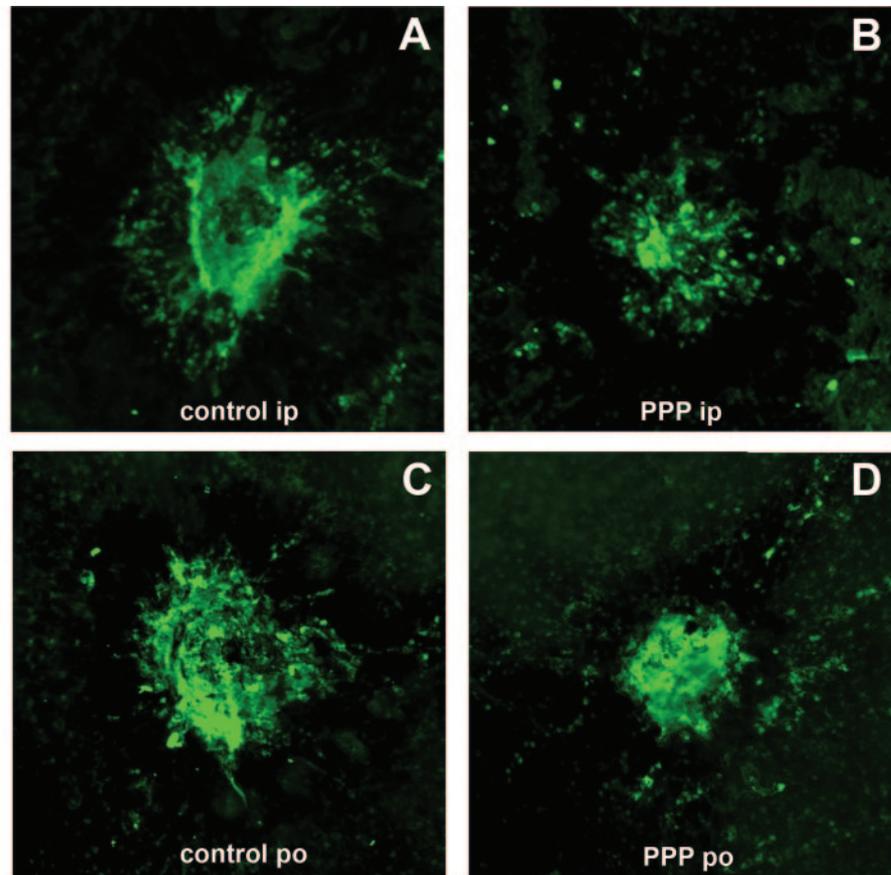
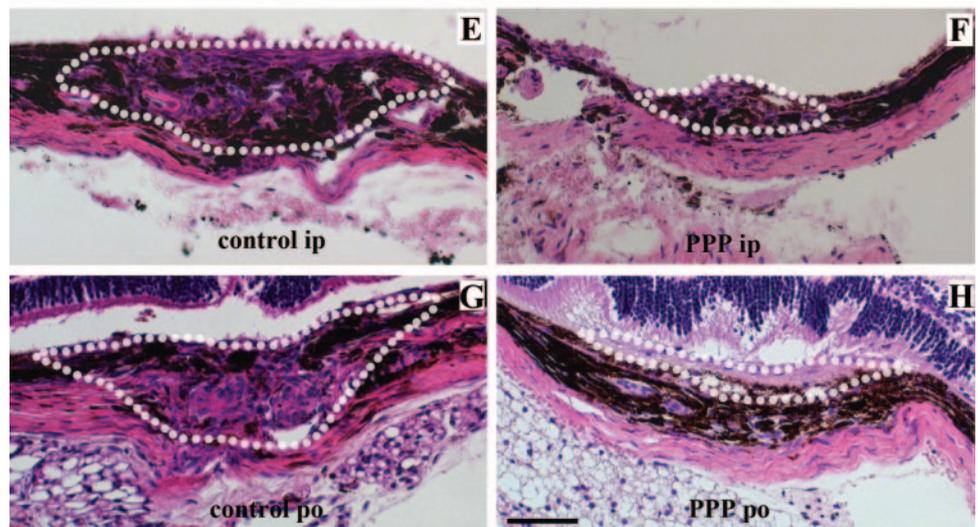


FIGURE 2. PPP suppressed the development of CNV lesions at sites of laser-induced rupture of Bruch's membrane, according to histology of CNVs after PPP treatment. Choroidal flatmounts were examined by fluorescence microscopy after immunostaining with isolectin B4 to identify vessels. Images of animals treated with DMSO (control) (A) compared with animals treated with 20 mg/kg/12 h IP of PPP (B) as well as animals treated with control food (C) and PPP-containing food (D) are shown. Hematoxylin-eosin-stained serial sections of choroids. Magnification, $\times 20$. In the control group (E), light microscopy at 14 days after krypton laser coagulation showed a domelike CNV complex that emerges through the ruptured Bruch's membrane and scattered RPE layer. After PPP treatment with 20 mg/kg/12 h IP (F), a marked thinning and fibrosis of the CNV complex was seen without apparent damage to the overlying neural retina. Similar differences in thickness of the CNV complex are seen in control group (G) and PPP group (H) in the peroral experiment.



VEGF Expression in the Choroidal and RPE Layers

In a separate *in vivo* experiment, in which q5 mice were fed with control food or food containing PPP, RPE-choroid-sclera eyecups from each mice were subjected to analysis of VEGF expression by Western blot analysis. VEGF protein expression, normalized to the GAPDH expression, in the PPP+laser group was significantly decreased compared with the laser group ($P = 0.005$) but also compared with the no-laser group ($P = 0.02$; Figs. 4A, 4B). VEGF protein expression was increased after laser injury (laser group) compared with that in the no-laser group, although the difference was not statistically significant ($P = 0.06$; Fig. 4C).

In the same experiment, we observed a statistically significant decrease in the levels of IGF-1R expression in the PPP+laser group, compared with that in the no-laser group ($P = 0.02$; Figs. 4A, 4B). In addition, we did not observe any significant change in the expression of IGF-1, as a result of the PPP treatment compared with the no-laser or laser groups.

DISCUSSION

To our knowledge, the present study is the first to reveal that an inhibitor of the IGF-1R downregulates the VEGF expression and CNV *in vivo*. In particular, we observed that the systemic treatment, either intraperitoneal or oral, with the IGF-1R inhib-

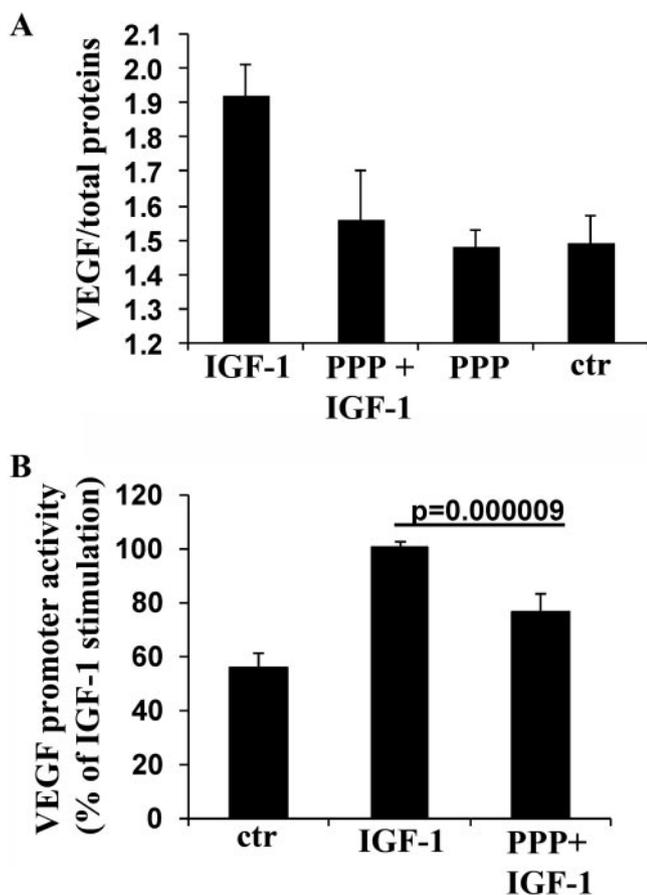


FIGURE 3. Effect of PPP on VEGF secretion and transcription in ARPE-19 cells. (A) Serum-depleted ARPE-19 cells remained untreated or were treated with IGF-1 (100 ng/mL), with or without 1 μ M PPP for 24 hours. The media were collected for ELISA of VEGF. The concentration of VEGF was normalized to protein content of the cells. The mean \pm SD of results of triplicates are shown. PPP reduced IGF-1-induced VEGF secretion significantly ($P < 0.05$). (B) The VEGF reporter gene construct was transfected into ARPE-19 cells. The cells were treated as described in (A), and luciferase activity was assayed. Luciferase activity was normalized to cell number as measured by XTT assay. The mean \pm SDs of luciferase activity of six different experiments are shown as relative values (100% represents IGF-stimulated condition). PPP decreased IGF-1-induced VEGF transcription significantly ($P = 0.000009$).

itor PPP caused an approximate 30% suppression of laser-induced CNV in C57BL/6J mice. Our results are consistent with those of Slomiany et al.,¹⁵ showing that IGF-1 induces secretion of VEGF in RPE cells (ARPE-19). In addition, we demonstrated that the IGF-1-induced VEGF production was blocked by PPP. Of note, the levels of laser-induced VEGF in the choroidal-RPE layers of PPP-treated mice were also decreased.

It has been known that IGF-1 participates in ocular neovascularization.³² Furthermore, it has been demonstrated in vitro that IGF-1 stimulates the proliferation of choroidal endothelial cells.³³ IGF-1 has also been shown to be involved in the degradation of basement membranes and proteolysis of extracellular matrix and migration.³⁴ Intravitreal injection of IGF-1 produces preretinal neovascularization in rabbits³² and generates microangiopathy resembling diabetic microangiopathy in pigs.³⁵ IGF-1 also increases RPE cell migration and proliferation in vitro.³⁶ Furthermore, IGF-1 induces upregulation of VEGF mRNA expression in fibroblasts,³⁷ which in turn stimulates endothelial cell proliferation.³⁸ Antagonism of IGF-1R with JB3, a long-acting IGF-1R peptide antagonist, suppresses retinal neovascularization by reducing the retinal endothelial cell response to VEGF.³⁷ All these results suggest that IGF-1 signaling has a permissive role in VEGF-induced neovascularization. Therefore, expression of IGF-1R and -1 in both endothelial and RPE cells in CNV implies a role for this growth factor and its receptor in the pathogenesis of neovascular AMD. Both cell types also appear to be important in this process.³⁹

Inhibition of IGF-1R activity with PPP may directly target the neovascularization process, through decreasing the endogenous angiogenic effect of IGF-1R signaling, and indirectly via attenuation of VEGF. Another possible mechanism behind the antiangiogenic effect of PPP may be associated with matrix metalloproteinase (MMP)-2. It is known that MMPs increase the bioavailability of VEGF⁴⁰ and that MMP-2 plays an important role in promoting CNV in vivo.^{26,41} MMP-2 and -9 were detected in surgically excised CNV membranes of patients with exudative AMD.⁴² IGF-1R has been shown to affect biosynthesis and activation of MMPs.⁴³ In a recent study, we observed that both the IGF-1-induced activation and expression of MMP-2 are repressed by PPP in a dose-dependant manner in cultured uveal melanoma cells.^{24,44} Furthermore, MMP-2 expression was downregulated by PPP in uveal melanoma xenografts in vivo.²⁴

We also observed a significant decrease in the levels of IGF-1R expression in choroidal tissues of PPP-treated mice.

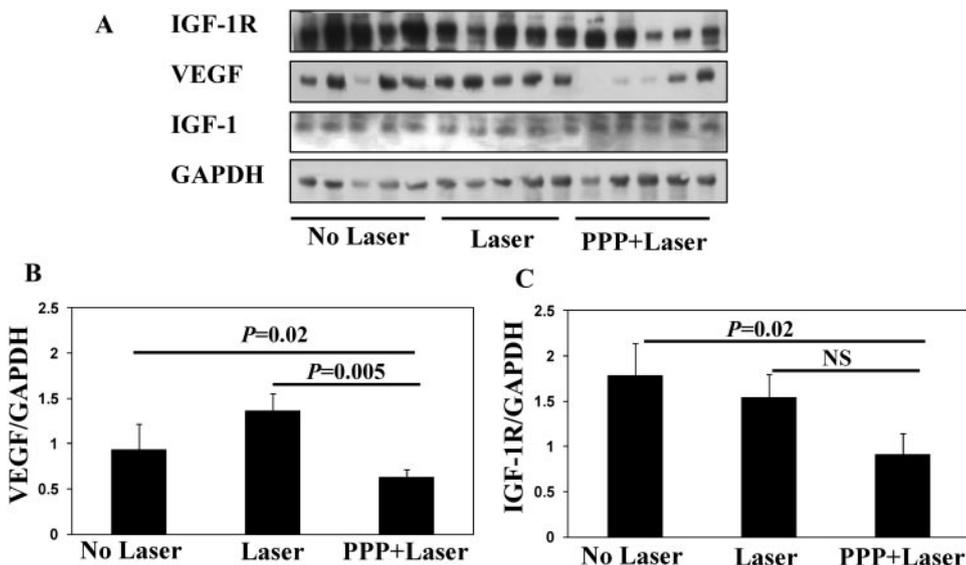


FIGURE 4. (A) VEGF, IGF-1, and IGF-1R expression in RPE/choroidal layer of control and PPP-treated mice, exposed and nonexposed to krypton laser photocoagulation. Western blot data with obtained from three different experiments ($n = 5$ mice for each group). (B) VEGF protein expression, normalized to the GAPDH expression, in the PPP+laser group was significantly decreased compared with the laser group, $P = 0.005$ and $P = 0.02$, respectively. Also the levels of VEGF protein expression were increased after laser injury (laser group) compared with the no-laser group, although the difference was not statistically significant ($P = 0.06$). (C) In the same experiment, the levels of IGF-1R expression in the PPP+laser group compared with both the laser (not significant) and the no-laser group, ($P = 0.02$) were determined.

This effect is consistent with that in tumor cells and tumor xenografts.²⁴ We did not detect a significant effect of PPP on the expression of IGF-1. That result was predictable, because PPP is a specific inhibitor of the receptor and does not target the ligand.

In vivo the inhibitory effect of systemically administered PPP on CNV area in vivo was limited to 30%. However, it is reasonable to expect that stronger effects of PPP can be achieved if the substance is administered intravitreally, since it does not have to pass the blood-retina barrier under such conditions. Such experiments are presently being planned. In contrast, system administration of PPP could still be a clinical option if considered in a combination therapy regimen.

Currently, all antiangiogenic agents used for AMD are applied intravitreally because of the risk of side effects (e.g., hypertension and stroke) when administered systemically.⁴⁵ Current anti-VEGF treatment regimens for neovascular AMD require multiple intravitreal injections each year. Therefore, it is a good rationale for identifying new antiangiogenic agents with either less invasive routes of administration or with a longer half-life or preferably both. Also, the current treatment options only have limited anti-angiogenic effects (and mainly targets vascular leakage) and do not eradicate the CNV lesion. Use of oral administration, as reported herein, could be an alternative strategy to combat the neovascular form of AMD, perhaps in conjunction with current treatments.

In summary, in the current study, PPP suppressed VEGF levels both in vitro and in the choroids in vivo and significantly reduced CNV. Although additional studies are necessary to explore the potential therapeutic role of PPP further, our current data suggest the potential for IGF-1R inhibitors in CNV treatment.

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