The Kinase Inhibitor PKC412 Suppresses Epiretinal Membrane Formation and Retinal Detachment in Mice with Proliferative Retinopathies

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PURPOSE. Platelet-derived growth factor (PDGF) is an important stimulatory factor for proliferative retinopathies. Expression of PDGF-B in the retinas of transgenic mice (hemizygous rho/PDGF-B mice) results in rapid-onset retinal detachment caused by proliferation of glial cells, endothelial cells, and pericytes, whereas expression of PDGF-AA (hemizygous rho/PDGF-A or PDGF-AA mice) causes slowly progressive retinal detachment from proliferation of glial cells. In this study, we investigated the effect in rho/PDGF-B and rho/PDGF-AA mice of several different receptor kinase inhibitors.

METHODS. Hemizygous rhoPDGF-B or homozygous rho/PDGF-A mice were treated orally with PKC412 (an inhibitor of PDGF, VEGF, and c-kit receptor kinases and several isoforms of PKC), PTK787 (an inhibitor of PDGF, VEGF, and c-kit receptor kinases), SU1498 (an inhibitor of VEGF receptor kinases), imatinib mesylate (an inhibitor of PDGF, c-kit, and v-abl receptor kinases), or vehicle, and at appropriate time points epiretinal membrane (ERM) formation and retinal detachment were quantified.

RESULTS. In either rho/PDGF-B or rho/PDGF-A mice, oral administration of PKC412 or PTK787, but not SU1498 or imatinib mesylate, significantly reduced ERM formation. PKC412 reduced the incidence of severe retinal detachments in both models and PTK787 did so in homzygous rho/PDGF-A mice.

CONCLUSIONS. These data indicate that PKC412 (and possibly PTK787) has appropriate activity and sufficient intraocular bioavailability after oral administration to prevent retinal detachment in models of proliferative retinopathy. PKC412 should be considered for treatment of vascular and nonvascular proliferative retinopathies in humans.

Epiretinal membrane (ERM) formation, a proliferation of cells in the retina resulting in sheets of cells and extracellular matrix that exert traction on the retina, is a common cause of visual impairment that occurs as part of several different disease processes. Moderate ERMs cause wrinkling and distortion of the retina, and when the macula is involved, results in metamorphopsia and mild to moderate decreased vision. Severe ERMs cause retinal detachment and severe visual loss and, unless corrected by vitreous surgery, can cause blindness.

In ischemic retinopathies, such as proliferative diabetic retinopathy (PDR), the ischemic retina releases vascular endothelial growth factor (VEGF)1,2 and other factors. Increased expression of VEGF is both necessary and sufficient for the development of retinal neovascularization,3–5 and, although other factors may participate,6 VEGF plays a central role. The new blood vessels lay down extracellular matrix and recruit glial cells and retinal pigmented epithelial (RPE) cells, resulting in scar tissue that can obscure the retina and/or detach it.7 The stimuli responsible for recruitment of RPE and glial cells by new blood vessels are not known with certainty, but several lines of evidence have implicated platelet-derived growth factor (PDGF). PDGF is a potent chemoattractant for retinal glia and RPE cells8,9 PDGF B-chain (PDGF-B) is produced by endothelial cells, and endothelial cell-derived PDGF-B is necessary for pericyte recruitment during vascular development.10 Transgenic mice in which the rhodopsin promoter drives expression of PDGF-B in photoreceptors (rho/PDGF-B mice) show development of ERMs consisting of glial cells, endothelial cells, and pericytes that cause traction retinal detachment within 2 to 3 weeks of the onset of transgene expression.11 The cellular components are similar to those in diabetic membranes and therefore, rho/PDGF-B mice provide a useful model of diabetic traction retinal detachment.

PDGF A-chain (PDGF-A) is produced by retinal ganglion cells and vascular cells, and during development PDGF-A stimulates migration of astrocytes into the retina from the optic nerve.12 The expression of PDGFs in the retina is reduced in adults, but retinal detachment results in increased production of PDGFs by RPE cells, and several lines of evidence have implicated PDGF-A in proliferative vitreoretinopathy (PVR), a disease process in which ERMs and traction retinal detachment occur after retinal reattachment surgery.15–21 In transgenic mice, in which the rhodopsin promoter drives expression of PDGF-A in photoreceptors (rho/PDGF-A mice), ERMs develop that consist solely of glial cells.12 Homozygous rho/PDGF-A mice have slowly progressive retinal detachment, and after detachment, RPE cells proliferate, resulting in subretinal membranes, and eventually in a funnel-shaped detachment.22 This model mimics many aspects of PVR. In this study, we sought to determine whether various receptor kinase inhibitors could prevent traction retinal detachment in these two proliferative retinopathy models.

MATERIALS AND METHODS

Transgenic mice
Mice were treated humanely in accordance with the ARVO Statement for the Use of Animals in Ophthalamic and Vision Research. The gen-
eration and characterization of rho/PDGF-A and -B transgenic mice has been described.11,23 Hemizygous rho/PDGF-B, line 1 mouse in a C57BL/6 background and homozgyous rho/PDGF-A, line 2 mice in a C57BL/6 background were each divided into five groups and, starting on postnatal day (P)7, were treated by gavage once a day with vehicle or containing 50 mg/kg PKC412, PTK787, SU1498, or imatinib mesylate. Rho/PDGF-B mice were killed at P12 to assess ERM formation by staining with Griffonia simplicifolia (GSA) lectin, which selectively stains vascular cells,24 or at P21 to assess for retinal detachment. These time points were selected based on a previous time course study.14 Homozygous rho/PDGF-A mice were killed at P40 to assess formation of ERMs by immunohistochemical staining for glial fibrillary acidic protein (GFAP) or at P50 to assess for retinal detachment. These time points were selected based on a previous study.25

**Drugs**

PKC412 is an inhibitor of the kinase activity of several isoforms of PKC, VEGF receptors, PDGF receptors, and c-kit, but not of receptors of several other growth factors that have been tested.23,26 PTK787 is an inhibitor of the kinase activity of VEGF receptors, PDGF receptors, and c-kit, but not of receptors of several other growth factors that have been tested.23,26 SU1498 is an inhibitor of the kinase activity of VEGF receptors.29 Imatinib mesylate is an inhibitor of the kinase activity of PDGF receptor kinase, c-kit, and vabl, but not of the receptors of several other growth factors.30

**Histochonometric Staining with GSA Lectin**

Rho/PDGF-B mice were killed and eyes were rapidly removed and frozen in optimal cutting temperature (OCT) embedding medium (Miles Diagnostics, Elkhart, IN). Ten-micrometer frozen sections were fixed with 4% paraformaldehyde for 30 minutes and washed with 0.05 M Tris-buffered saline (TBS; pH 7.6). Slides were incubated in methanol/H2O2 for 10 minutes at 4°C, washed with 0.05 M TBS and incubated for 30 minutes in 10% normal porcine serum. Slides were incubated 2 hours at room temperature with biotinylated GSA lectin (Vector Laboratories, Burlingame, CA) and after rinsing with 0.05 M TBS, they were incubated with avidin coupled to peroxidase (Vector Laboratories) for 45 minutes at room temperature. After a 10-minute wash in 0.05 M TBS, slides were incubated with diaminobenzidine (Research Genetics, Huntsville, AL), to produce a brown reaction product, and were counterstained with eosin.

**Immunohistochemical Staining for GFAP**

Immunohistochemical staining of retinas for GFAP labels astrocytes and activated Müller cells. Homozygous rho/PDGF-A mice were killed and eyes were frozen in OCT compound. Ten-micrometer frozen sections were fixed with 4% paraformaldehyde for 30 minutes, washed with 0.05 M TBS, incubated in methanol/H2O2 for 10 minutes at 4°C, and washed with 0.05 M TBS. Specimens were blocked with 10% normal goat serum (NGS) for 0.05 M TBS for 30 minutes at room temperature and then incubated with 1:500 rabbit anti-bovine GFAP in 1% NGS and 0.05 M TBS and incubated in biotinylated goat anti-rabbit antibody for 30 minutes. After washing, the slides were incubated in streptavidin-phosphatase and developed with a red stain (Histomark Red, Kirkegaard and Perry, Gaithersburg, MD), according to the manufacturer’s instructions, and counterstained with hematoxylin. Sections were dehydrated and mounted in acrylic medium (Cytoseal; Stephens Scientific, Cornwall, NJ).

**Quantitative Assessments**

For quantitative assessments, 10-µm serial sections were cut through an entire eye starting with sections that included the iris root on one side of the eye and proceeding to the iris root on the other side. Every 10th section, roughly 100 µm apart, was stained with GSA (rho/ PDGF-B mice) or anti-GFAP (homozygous rho/PDGF-A mice). Sections were examined by microscope (Axioskop; Carl Zeiss Meditech, Thornwood, NY), with the examiner masked to treatment group. For assessment of the amount of ERM, images were digitized with a three-color charge-coupled device video camera and a frame grabber. Image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was used to delineate GSA- or GFAP-stained cells in the retina and to measure their area. The mean of the measurements from each eye was used as a single experimental value. For assessment of retinal detachment, sections were examined and graded as to the presence of partial or total retinal detachment. If all stained sections showed total retinal detachment, the eye was graded as having total retinal detachment. If any of the sections showed at least a partial retinal detachment, but all sections did not show total retinal detachment, the eye was graded as having partial retinal detachment. If none of the sections showed any retinal detachment, the eye was graded as having no retinal detachment.

**Statistical Analysis**

For analysis of area measurements, data were analyzed using both a generalized linear model with generalized estimating equations (GEE) and an analysis of variance (ANOVA) model.31 The generalized linear model with GEE is analogous to the ANOVA model. The primary difference is that it allows for correlated data from right and left eyes to be included directly in the analysis by modeling the correlation, whereas the ANOVA model requires the data to be independent, and hence right and left eye data must be averaged before analysis. Findings with respect to significant treatment comparisons were the same for both analyses. Probabilities from the generalized linear model with GEE are reported. For comparisons of the presence of retinal detachment between vehicle-treated eyes and eyes in the other treatment groups, data were analyzed by logistic regression with GEE to account for correlation between eyes. A Bonferroni correction was used to adjust for multiple comparisons in all the analyses. Specifically, there were four active treatments compared with the vehicle; thus, \( P = 0.05/4 = 0.0125 \) was required for statistical significance.

**RESULTS**

**Effect of PKC412 and PTK787 on ERM Formation**

Starting at P7, hemizygous rho/PDGF-B mice were given vehicle or containing 50 mg/kg PKC412, PTK787, SU1498, or imatinib mesylate once a day by gavage. Untreated rho/ PDGF-B mice consistently had prominent ERMs by P12 that are best illustrated by staining retinal sections with GSA,11 and consistent with those previous results, extensive GSA-stained ERMs were present in the eyes of mice treated with vehicle (Figs. 1A, 1B). In contrast, eyes of mice treated with PKC412 or PTK787 had little ERM formation (Figs. 1C–F). Eyes of mice treated with SU1498 or imatinib mesylate had extensive ERMs (Figs. 1G–J), similar to that in vehicle-treated eyes. Measurement of the area of GSA staining by image analysis showed significantly smaller areas in PKC412- and PTK787-treated mice compared with vehicle-treated mice (Fig. 2), whereas mice treated with SU1498 or imatinib mesylate showed no significant difference.

**Effect of PKC412 on Severe Retinal Detachments**

At P21, vehicle-treated rho/PDGF-B mice had extensive formation of ERMs, with multiple layers of ectopic cells in the inner retina, and more than 80% of eyes had folding of the outer retina and retinal detachment, with total funnel-shaped detachments in approximately one third of eyes (Figs. 3A, 3F). Mice treated by gavage with 50 mg/kg PKC412 or PTK787 between P7 and P21 had moderate ERM formation and roughly 50% of the eyes had a normal-appearing outer retina (Figs. 3B, 3C, 3F). Only 10% of eyes of mice treated with PKC412 and 15% of those of mice treated with PTK787 had total retinal detachments, compared with 35% of eyes in mice treated with vehicle (Fig. 3F). Mice treated with SU1498 or imatinib mesylate had extensive ERM formation and nearly 90% had at least partial total retinal detachment.
retinal detachment. Sixty percent of eyes of mice treated with SU1498 and 30% of those mice treated with imatinib mesylate had total retinal detachment (Fig. 3F).

Because of the many experimental groups requiring multiple comparisons, the decrease in total retinal detachments in the PKC412 and PTK787 groups was not considered statistically significant. An independent experiment was performed to compare treatment with PKC412 with treatment with vehicle.

Effect of PKC412 and PTK787 on ERM and Retinal Detachment

Homozygous rho/PDGFB mice (rho/PDGFBAA mice) have glial ERMs that are slowly progressive and often result in traction retinal detachment between 1 and 2 months of age. At P40, eyes of mice treated with vehicle had a thick layer of GFAP-stained cells on the surface of the retina and within the inner nuclear layer (Fig. 4A). Eyes of mice treated with PKC412 had a layer of glial cells on the surface of the retina, but none or few in the inner nuclear layer, and the total area of GFAP staining per section was significantly less than that in vehicle-treated eyes (Figs. 4B, 4F). Eyes of mice treated with PTK787 had a layer of GFAP-positive cells on the surface of the retina and occasional clumps in the inner nuclear layer, but the total area of GFAP staining per section was significantly less than that in vehicle-treated eyes (Fig. 4F). Many of the eyes of mice treated with imatinib mesylate appeared to have ERMs to a lesser degree (Fig. 4E), but the area of GFAP-positive cells when analyzed with multiple comparisons did not achieve a statistically significant difference from vehicle-treated eyes (Fig. 4F).

At P50, most of the eyes of vehicle-treated mice (>60%) showed total, funnel-shaped detachments (Figs. 5A, 5F), whereas most of the eyes of PKC412- or PTK787-treated mice showed no detachments (Figs. 5B, 5C). Only 10% to 15% of eyes in the PKC412 or PTK787 groups had total retinal detachments, which is significantly less than in the vehicle-treated group (Fig. 5F). There was no decrease in total retinal detachments in mice treated with SU1498 compared with those treated with vehicle (Figs. 5D, 5F), and although there were
FIGURE 3. Rho/PDGF-B mice treated with PKC412 had fewer severe retinal detachments than mice treated with vehicle. P7 hemizygous rho/PDGF-B mice were given vehicle or vehicle containing 50 mg/kg PKC412, PTK787, SU1498, or imatinib mesylate once a day by gavage. At P21, ocular sections were stained with GSA lectin, which selectively stains vascular cells. Total retinal detachments were common in vehicle-treated mice (A) or mice treated with SU1498 (D) or imatinib mesylate (E), occurring in more than one third of the eyes in each of those groups (F). In mice treated with PKC412 (B) or PTK787 (C), most eyes showed no detachment or small focal detachments, with total detachment occurring in approximately 10% (F). Because of the large number of treatment groups requiring multiple comparisons, these differences did not achieve statistical significance, and therefore an independent experiment was performed to compare PKC412 and vehicle treatment. Logistic regression with general estimating equations to account for correlation between eyes demonstrated that there were significantly fewer total retinal detachments in eyes of mice treated with PKC412 than in mice treated with vehicle (G). Bar, 100 μm.

FIGURE 4. PKC412 and PTK787 reduced ERM formation in homozygous rho/PDGF-A mice. P7 homozygous rho/PDGF-A mice were given vehicle or vehicle containing 50 mg/kg PKC412, PTK787, SU1498, or imatinib mesylate once a day by gavage. At P40, serial ocular sections were stained with GFAP, and the stained area was measured in each eye. GFAP robustly stained ERMs in vehicle-treated eyes (A). There appeared to be less ERM in eyes treated with PKC412 (B) and PTK787 (C) and in some eyes treated with imatinib mesylate (E), but not in eyes treated with SU1498 (D). Measurement of the area of GFAP-stained ERMs by image analysis showed significant reduction in eyes of mice treated with PKC412 or PTK787 compared with those treated with vehicle (F). Statistical comparisons were made with a generalized linear model with generalized estimating equations (GEE) using the average area per eye. The correlation between eyes is accounted for by GEE. Probabilities are for the difference compared with vehicle-treated mice and because there are multiple comparisons, the cutoff for statistical significance is \( P = 0.0125 \). Bar, 100 μm.
fewer severe detachments in mice treated with imatinib mesylate, the difference did not meet the rigorous criterion required for statistical significance, given the need for multiple comparisons among the five groups (Figs. 5E, 5F).

DESCRIPTION

We have demonstrated that hemizygous rho/PDGF-B transgenic mice have vascular ERMs and traction retinal detachments similar to those in patients with severe ischemic retinopathies. In homozygous rho/PDGF-A mice, avascular ERMs and traction retinal detachments develop that are similar to those in patients with PVR. By visual inspection of representative sections, imatinib mesylate appeared to decrease the degree of ERM formation in rho/PDGF-A mice, but measurement of the area of ERM by image analysis showed that the decrease was not statistically different from that in vehicle-treated eyes. This does not mean that imatinib mesylate does not decrease ERM formation in rho/PDGF-A mice, and it would make biological sense if it did, because it blocks PDGF receptor kinase activity in vitro. It just means that with the experimental design that we used, which includes multiple comparisons, we have not shown that imatinib mesylate decreases ERM formation in rho/PDGF-A mice, whereas we have shown that PKC412 and PTK787 decrease ERM formation and therefore are superior.

In rho/PDGF-B mice, ERM formation and retinal detachment occur more aggressively than in rho/PDGF-A mice. Therefore, it is not surprising that PKC412 and PTK787 showed strong inhibition of retinal detachment in rho/PDGF-A mice, which was statistically significant, even with an experimental design requiring multiple statistical comparisons. In contrast, using the same experimental design, neither PKC412 nor PTK787 showed statistically significant inhibition of retinal detachment in rho/PDGF-B mice. We used a simplified design and found that oral administration of PKC412 clearly signifi-
cantly inhibited retinal detachment in rho/PDGF-B mice. We did not perform additional experiments with PTK787, and so we cannot say for certain that it inhibits retinal detachment in rho/PDGF-B mice, although we suspect that this is the case.

Although we did not show that the agents exert their effect on ERM formation by inhibition of PDGF signaling, this is a reasonable inference, because two of the three PDGF kinase inhibitors showed strong inhibitory activity, and the third showed a trend. Several lines of evidence have suggested that PDGF is an important stimulus for proliferative retinopathies. It is a potent mitogen and chemotactant for RPE cells, retinal glia, and pericytes, and expression of PDGF in the eye is upregulated after retinal detachment.11–15 Elevated levels of PDGF-AB have been demonstrated in the vitreous of patients with proliferative diabetic retinopathy (PDR) and PDGF-A, PDGF-B, PDGFαR, and PDGFβR have been localized to ERMs obtained during surgery in patients with PDR or PVR.16,17 When injected into the vitreous cavity of rabbits, cells that are unable to respond to PDGF have greatly reduced ability to cause PVR formation in the animals.18 However, scatter photocoagulation has limitations. It exacerbates macular edema, and the eye must have clear media for it to be applied. A drug treatment that could be used in conjunction with scatter photocoagulation would be a great benefit. There are several features of a retinal detachment and/or its initial management that prevent PVR from occurring.19 Inhibition of PDGF signaling is sufficient to completely prevent formation of ERMs and prevents redetachment to be extremely valuable. The results of our study suggest that clinical trials should be considered to determine whether PKC412 (and possibly PTK787) would be beneficial in patients with proliferative retinopathies.

References

3. Okamoto N, Tohe T, Hackett SF, et al. Transgenic mice with photoreceptor-specific expression of platelet-derived growth factor receptor signaling prevent PVR in a rabbit model.21

Thus, PDGFs have been implicated in vascular proliferative retinopathies such as those that occur in diabetic retinopathy and other ischemic retinopathies and in avascular proliferative retinopathies such as PVR. Currently, scatter photocoagulation is the only treatment for retinal neovascularization in patients with diabetic retinopathy or other ischemic retinopathies. If patients with high risk retinal neovascularization are not treated, a poor outcome almost always occurs, and frequently it can be prevented by scatter photocoagulation.20 However, scatter photocoagulation has limitations. It exacerbates macular edema, and the eye must have clear media for it to be applied. A drug treatment that could be used in conjunction with scatter photocoagulation would be a great benefit. There are several features of a retinal detachment and/or its initial management that predict a high risk of PVR, but there is no known effective treatment to prevent it. Drug treatment that reduces formation of ERMs and prevents redetachment would be extremely valuable. The results of our study suggest that clinical trials should be considered to determine whether PKC412 (and possibly PTK787) would be beneficial in patients with proliferative retinopathies.