Periocular Injection of Microspheres Containing PKC412 Inhibits Choroidal Neovascularization in a Porcine Model

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PURPOSE. Oral administration of PKC412, a kinase inhibitor that blocks several isoforms of protein kinase C (PKC) and receptors for vascular endothelial growth factor (VEGF), platelet-derived growth factor, and stem cell factor, inhibits ocular neovascularization in a murine model. The purpose of this study was to determine whether sustained local delivery of PKC412 in a human-sized eye inhibits choroidal neovascularization (CNV).

METHODS. Laser photocoagulation was used to rupture Bruch’s membrane in young domestic pigs, and then a periocular injection of control microspheres or microspheres containing 25% or 50% PKC412 was given. After 10 days the integrated area of CNV at Bruch’s membrane rupture sites was measured by image analysis. The levels of PKC412 in choroid, retina, and vitreous were measured either 10 or 20 days after periocular injection of 50% PKC microspheres or at 20 days after injection of 25% PKC microspheres.

RESULTS. The areas of CNV at Bruch’s membrane rupture sites were significantly smaller in eyes that received a periocular injection of microspheres containing 25% (P = 0.0042) or 50% (P = 0.0012) PKC412 than those in eyes injected with control microspheres. Ten days after periocular injection of 50% PKC412 microspheres, PKC412 was detected in the choroid, but not in the retina or vitreous. Twenty days after periocular injection of 50% PKC412, high levels of PKC412 were measured in the choroid, vitreous, and retina. Levels were lower but still substantial in all three compartments 20 days after periocular injection of 25% microspheres.

CONCLUSIONS. Sustained local delivery of PKC412 provides a promising approach for treatment of CNV. (Invest Ophthal Vis Sci. 2003;44:4989–4993) DOI:10.1167/iovs.03-0600

Although multiple stimuli may be involved in the development of CNV, VEGF seems to play a particularly important role. Increased levels of VEGF have been demonstrated in RPE cells in association with CNV,1–4 and agents that block signaling through VEGF receptors5 or sequester VEGF6,7 suppress the development of CNV. These preclinical studies predict that antagonizing VEGF is a good approach for treatment of CNV, and preliminary results in clinical trials suggest that this prediction may be correct8 (Rosenfeld PJ, et al. JOVS 2003;44: ARVO E-Abstract 970). Kinase inhibitors, such as PKC412, provide an efficient way to block signaling through VEGF receptors.9,10 Because PKC412 blocks both VEGF receptors 1 and 2, in addition to neutralizing the effects of VEGF, it also blocks the effects of other members of the VEGF family, such as placental growth factor, that may also play a role. Also, PKC412 is a small molecule, allowing it to be delivered by routes other than intravitreal injection, which carries risk of endophthalmitis and retinal detachment. A potential disadvantage is that the high homology among receptor tyrosine kinases makes it difficult to develop highly specific kinase inhibitors. In addition to blocking VEGF receptors, PKC412 blocks the receptors for PDGF and stem cell factor and inhibits several isoforms of PKC.9 There is always the possibility of unintended unfavorable consequences from one of these other activities. But it is also possible that these other activities are fortuitous, and in fact PDGFs have been implicated as contributors to proliferative retinopathies,11,12 PKCs contribute to vascular proliferation and leakage,13,14 and recently it has been shown that erythropoietic stem cells may contribute to neovascularization.15

In a phase 2 clinical trial, oral administration of PKC412 was found to decrease retinal thickening significantly in patients with macular edema due to diabetic retinopathy (Campochiaro PA, et al. JOVS 2003;44:ARVO E-Abstract 4286). However, some patients experienced nausea and vomiting, which generally was not serious, but still unpleasant. Also, a small number of patients showed elevation of liver enzymes, which required careful monitoring. These adverse effects could be avoided by sustained local delivery of PKC412. In this study, we investigated the effect of periocular injection of microspheres containing PKC412. We used a pig model of CNV, because the pig eye is similar to the human eye in size and thickness of the sclera.

MATERIALS AND METHODS

PKC412 and poly(d,l lactide-co-glycolide)glucosue are produced by Novartis Pharma (Basel, Switzerland). Poly(vinylalcohol) (Mowiol 4-88)

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4989
was purchased from Clariant (Frankfurt, Germany). An aqueous vehicle containing sodium carbomethylcellulose, poloxamer 188 (Pluronic F68; BASF, Ludwigshafen, Germany) and mannitol was used for suspension of microspheres.

Manufacturing of PKC412 Microspheres: 25% and 50% Loading

A solution of PKC412 drug substance and poly(d,l lactide-co-glycolide) glucose in methylene chloride was emulsified with a 1.5% aqueous solution of poly(vinylalcohol) and fed into a stirred tank reactor containing 0.5% aqueous solution of poly(vinylalcohol). The resultant emulsion was heated to 42°C with stirring for 30 minutes and then cooled to room temperature. The microspheres were allowed to sediment, the aqueous supernatant was removed, and the microspheres were washed twice by suspending in water, heating to 42°C with stirring for 30 minutes, sedimenting, and pouring off the supernatant. The microspheres were collected with a 5-mm filter, dried in a vacuum, sieved with a 140-mm filter, and sterilized in bulk by gamma irradiation (30 ≥ 2 kGy).

Characterization of Microspheres

Microspheres were dissolved in acetonitrile-methanol, and the polymer fraction was precipitated with an aqueous buffer. The amount of PKC412 in the liquid phase was measured by a standard HPLC method. Particle size was determined by the amount of defraction of a beam of laser light passed through an aqueous suspension of microspheres. Residual methylene chloride was determined by headspace gas chromatography of a heated solution of microspheres in dimethylformamide.

Rupture of Bruch's Membrane and Periocular Injections in Pigs

Four-week-old female Yorkshire pigs were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research in a protocol approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. The pigs were given 1% to 4% halothane by mask inhalation and then intubated. Anesthesia was maintained by inhalation of 1% to 3% halothane, 2% O2, and 1% NO2. During anesthesia, heart rate and O2 saturation were monitored by pulse oximetry. Pups were diluted with 1% tropicamide, and a fundus contact lens was placed on the cornea. The same approach was used to rupture Bruch's membrane as that previously described in mice.11 In preliminary experiments, a portable diode laser with a slit lamp delivery system was used to rupture Bruch's membrane in several locations, using a 75-mm spot size, 0.1-second duration, and several different power settings. As previously observed in mice, rupture of Bruch's membrane was accompanied by formation of a vaporization bubble. With our laser, the lowest power that consistently caused rupture of Bruch's membrane, as judged by the formation of a bubble, was 400 mW, and therefore this power setting was used. It should be noted that the lowest power setting that consistently causes rupture of Bruch's membrane may vary with different lasers, and it is important to perform the titration described earlier. To investigate the effect of PKC-containing microspheres, Bruch's membrane was ruptured in eight locations equidistant from and encircling the optic nerve in each eye of four pigs. After completion of the laser, two pigs received a periocular injection of 1.0 mL containing 100 μg of 25% PKC412 and two pigs received periocular injection of 1.0 mL containing 100 mg of 50% PKC412 in one eye. All four pigs received a periocular injection of 1.0 mL of placebo microspheres in the fellow eye. After 10 days, the pigs were killed and eyes were removed and frozen in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Torrance, CA).

Histopathologic Assessment of CNV

Frozen serial sections (10 μm) were cut through the entire extent of each burn and histochemically stained with Griffonia simplicifolia lectin B4 (GSA; Vector Laboratories, Burlingame, CA), which selectively stains vascular cells. A dye (HistoMark Red; Kirkegaard and Perry, Cabin John, MD) was used to stain the reaction product red, allowing it to be distinguished from melanin. Some slides were counterstained with hematoxylin (Sigma Diagnostics, St. Louis, MO). Sections were examined by microscope (Axioskop; Carl Zeiss Meditec, Inc., Thornwood, NY), and images were digitized with a three charge-coupled device (CCD) video camera (IK-TU40A; Toshiba, Tokyo, Japan) and frame grabber. Image-analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was used to delineate and measure the area of GSA-stained blood vessels in the subretinal space on each slide. Area measurements were made for all sections on which some of the lesion appeared and added together to give the integrated area of CNV at each Bruch's membrane rupture site, as previously described.10 If even one section was of such poor quality that the area of CNV could not be measured, then that lesion was discarded.

Measurement of Tissue and Plasma Levels of PKC412

PKC412 was measured by HPLC in tissues dissected from three porcine eyes: one, 10 days after periocular injection of 1.0 mL of 50% PKC412 microspheres; one, 20 days after periocular injection of 50% PKC412 microspheres; and one, 20 days after periocular injection of 1.0 mL of 25% PKC412 microspheres. After death, the eyes were rapidly removed and the vitreous, retina, and RPE-choroid were dissected and frozen. Plasma was also obtained and frozen.

Results

The characteristics of 25% and 50% PKC412 microspheres are listed in Table 1. Periocular injection of microspheres alone (placebo) or microspheres containing 25% or 50% PKC412 caused mild conjunctival injection that was similar among the three groups. There were no discernible signs of inflammation or irritation. Ten days after rupture of Bruch’s membrane and periocular injection of the microspheres, pigs were examined just before (Figs. 1A, 1C) or just after (Fig. 1B) death. The microspheres appeared as bulges beneath the conjunctiva. Gross pathologic examination of the eyes showed collections of microspheres on the outside of the sclera occupying approximately 1 quadrant of the eye extending from a few millimeters posterior to the limbus to the optic nerve (Figs. 1D–F). All the eyes were similar in appearance, with no gross signs of inflammation.

There were large areas of CNV at Bruch’s membrane rupture sites in eyes that had received a periocular injection of placebo microspheres (Figs. 1G, 1J). In contrast, areas of CNV at rupture sites were noticeably smaller in eyes treated with microspheres containing 25% (Figs. 1H, 1K) or 50% PKC412 (Fig. 1I, 1L). Measurement of the integrated area of CNV at Bruch’s membrane rupture sites by image analysis with the investigator masked with respect to treatment group, showed significantly smaller areas of CNV at rupture sites in eyes treated with 25% or 50% PKC412 microspheres, compared with eyes treated with placebo microspheres (Fig. 2).

Ten days after periocular injection of 100 mg of microspheres containing 50% PKC412, levels of PKC412 were below the level of detection in the plasma, vitreous, and retina, but

Table 1. Characterization of PKC412 Microspheres

<table>
<thead>
<tr>
<th>Parameter</th>
<th>25%</th>
<th>50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size Xa (μm)</td>
<td>67.7</td>
<td>42.5</td>
</tr>
<tr>
<td>Loading (%)</td>
<td>22.5</td>
<td>45.9</td>
</tr>
<tr>
<td>Loading after gamma irradiation (%)</td>
<td>22</td>
<td>45.7</td>
</tr>
<tr>
<td>Residual amount of dichloromethane (%)</td>
<td>1.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>
were measurable in the choroid (Table 2). Twenty days after periocular injection of 100 mg of 50% PKC412 microspheres, there were high levels of PKC412 in choroid, vitreous, and retina. In an eye given a periocular injection of 100 mg of 25% PKC412 microspheres, the levels of PKC412 in each of the three compartments 20 days after the injection were much lower than those in the eye injected with 50% microspheres, but were still substantial. With either dose, there was no detectable PKC412 in plasma.

TABLE 2. Ocular Levels of PKC412 after Periocular Injection of Microspheres Containing PKC412

<table>
<thead>
<tr>
<th>10 Days</th>
<th>20 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%†</td>
<td>25%</td>
</tr>
<tr>
<td>Vitreous (ng/mL)</td>
<td>0</td>
</tr>
<tr>
<td>Retina (ng/retina)</td>
<td>0</td>
</tr>
<tr>
<td>Choroid (ng/choroid)</td>
<td>0.572</td>
</tr>
<tr>
<td>Plasma (ng/mL)</td>
<td>0</td>
</tr>
</tbody>
</table>

PKC412 levels are reported for three pigs' eyes, one 10 days after periocular injection of 50% microspheres, one 20 days after periocular injection of 25% microspheres, and one 20 days after periocular injection of 50% microspheres.

* Days after injection.
† Percentage of PKC412 in microspheres.
DISCUSSION

In this study, we demonstrated that periocular injection of microspheres containing PKC412 caused significant suppression of the development of CNV at rupture sites in Bruch’s membrane in pigs. Ten days after injection of microspheres containing 50% PKC412, PKC412 was detectable in the choroid, but not the retina or vitreous. Twenty days after injection of microspheres containing 50% PKC412, levels were quite high in the choroid and lower, but still substantial, in the vitreous and retina. Twenty days after injection of 25% PKC412 microspheres, levels of PKC412 in choroid, vitreous, and retina were lower than those after injection of 50% PKC412 microspheres, but still well above the level of detection. This suggests that the microspheres were providing sustained delivery of PKC412, with levels increasing between 10 and 20 days after injection and were able to suppress the development of CNV. This provides proof of concept for local transscleral delivery of PKC412 for treatment of CNV.

Local delivery of antiangiogenic agents to the eye is an appealing approach for treatment of ocular neovascularization. One method to achieve this objective is to inject drugs into the vitreous cavity, an approach that is being tested in clinical trials for delivery of an aptamer that binds VEGF and an anti-VEGF antibody fragment8 (Rosenfeld PJ, et al. IOVS 2003;44:ARVO E-Abstract 970). With this approach, high drug levels can be achieved rapidly in both the retina and the choroid, but clearance is also quite rapid, requiring that injections be repeated every few weeks. This is inconvenient for the patient and the doctor, but more important, each injection carries some risk of endophthalmitis and retinal detachment. Whether the magnitude of this risk is acceptable or a serious drawback should be determined in ongoing clinical trials. Another approach is to implant a nonbiodegradable device into the vitreous cavity to slowly release drug over a prolonged period. This technique was successfully used to deliver ganciclovir for the treatment of CMV retinitis17 and is currently being tested for intraocular delivery of dexamethasone for macular edema (Kuppermann BD, et al. IOVS 2003;44:ARVO E-Abstract 4289).

The risks of repeated intravitreal injections or surgical procedures may be acceptable if these approaches have far superior efficacy to other modes of delivery. Transscleral delivery of agents does not require penetration of the eye and disruption of the vitreous and therefore is theoretically safer, but little is known about what sort of drug levels can be achieved in the various intraocular compartments. It has been suggested that transscleral delivery of drugs is not feasible, because the rapid blood flow in the choroid would simply carry drugs away. The present study demonstrates that this theoretical concern is not a major problem, because a lipophilic drug, PKC412, is able to penetrate the sclera, achieve high levels in the choroid, and significantly suppress CNV at Bruch’s membrane rupture sites. Recently, we have demonstrated that periocular injection of adenoviral vectors encoding pigment epithelium-derived factor (PEDF) or soluble VEGF receptor 1 (Flt-1), results in transduction of pericellular cells and transscleral penetration of the proteins into the eye, resulting in suppression of CNV.20,21 Therefore, even proteins of 50 or 79 kDa can penetrate the sclera and achieve therapeutic levels in the choroid. These data suggest that transscleral delivery of agents, particularly with a sustained delivery strategy, is a viable approach for treatment of choroidal diseases. Currently, periocular injection of an angiostatic steroid is being tested in clinical trials as a treatment for neovascular age-related macular degeneration, and preliminary results suggest some benefit.22

Detectable levels of PKC412 were also obtained in the retina and vitreous 20 days after periocular injection of PKC412 microspheres, suggesting the possibility that transscleral delivery can also be considered for treatment of retinal neovascularization. This is not the case for PEDF and soluble Flt-1, which after periocular gene transfer do not penetrate into the retina in sufficient amounts to inhibit retinal neovascularization,20,21 but small molecules like PKC412 may be able to do so. Additional studies are needed to explore this possibility, thoroughly investigate the pharmacokinetics, and determine the duration of PKC412 levels in the eye after periocular injection of PKC412 microspheres.

References


