Factor VII–Verteporfin for Targeted Photodynamic Therapy in a Rat Model of Choroidal Neovascularization

Fang Lu, Zhiwei Hu, John Sinard, Alan Garen, and Ron A. Adelman

PURPOSE. To study the efficacy and safety of factor VII (FVII)–verteporfin for targeted photodynamic therapy (TPT) compared with nontargeted photodynamic therapy (PDT) in a rat model of choroidal neovascularization (CNV). FVII–verteporfin binds tightly and specifically to tissue factor, which is expressed on endothelial cells of CNV but not normal vasculature.

METHODS. Multiple CNV lesions were induced by laser photocoagulation of the retina in Brown-Norway rats. After 3 weeks, the rats were injected intravenously with FVII–verteporfin (0.5 and 1.0 mg/m²) or Visudyne (6.0 mg/m², QLT Inc., Vancouver, BC, Canada). Randomly selected lesions were treated with a 689-nm laser 30 or 60 minutes later. The lesions were evaluated by fluorescein angiography and histopathology.

RESULTS. The rats treated with Visudyne PDT showed leakage in 75% of the CNV lesions on day 7 and 100% of lesions on day 14. The rats treated with FVII–verteporfin TPT at a dose of 0.5 mg/m² showed leakage in 33% and 56% of the CNV lesions on days 7 and 14, respectively. When the dose was increased to 1.0 mg/m² for TPT, leakage was detected in 25% and 23% of the CNV lesions on days 7 and 14, respectively. No ocular side effect was detected by histopathologic evaluation.

CONCLUSIONS. The frequency of leakage in CNV lesions was significantly reduced using FVII–verteporfin TPT compared with PDT. The efficacious dose with FVII–verteporfin was approximately 10% of the dose usually used in nontargeted Visudyne PDT. Using FVII–verteporfin for TPT may improve the efficacy and safety of PDT for treating choroidal neovascularization.

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Induction of CNV Membranes

All studies were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of Yale University’s Institutional Animal Care and Use Committee on Animal Research. Brown-Norway rats (250–300 g) were purchased from Harlan Sprague-Dawley, Inc., (Indianapolis, IN) and anesthetized with 0.2 to 0.3 mL of a 1:1 mixture of ketamine (100 mg/mL) and xylazine (20 mg/mL) by intraperitoneal injection. The pupils were dilated with topical application of 2.5% phenylephrine and 1.0% tropicamide. An argon green laser (Ultima model 2000 SE; Coherent Medical Laser, Palo Alto, CA) was used to induce six or seven laser-spot lesions in each eye around the optic nerve. The wavelength was 532 nm, and the spot size was 100 μm. Power delivered ranged from 160 to 170 mW, applied for 0.1 second. A bubble or a small subretinal hemorrhage (diameter, <1 mm) at the laser spot indicated rupture of Bruch’s membrane. The formation of CNV was confirmed by fluorescein angiography and fundus photography. Because the endothelial cells in the arborizing neovascularization do not have the barrier function as mature endothelial cells, the pathologic neovascular membrane usually leaks fluid, proteins, and lipids. Therefore, the fluorescein molecules leak from the CNV lesions into the neurosensory, subretinal, and retinal pigment epithelial (RPE) layers of the retina. Fluorescein angiography 3 weeks after laser showed hyperfluorescence in the early phase, with late leakage at the site of the laser injury (Fig. 1A).
Synthesis and Conjugation of fVII to Verteporfin
Chinese Hamster Ovary (CHO) cells were stably transfected with the expression plasmid pcDNA3.1 encoding mouse fVII/S-peptide (D14N)/8x histidine (fVII-S-His, abbreviated as fVII). The cDNA was amplified by PCR using a pcDNA3.1(+) DNA encoding mVII(K341A)/hlgG1Fc as a template. The S-peptide sequence is derived from the sequence of bovine RNase, and the His-tag sequence is an affinity tag for purification. Briefly, the CHO cells were grown in serum-free medium, and the encoded fVII-S-His protein was purified from the medium by chromatography on Ni-NTA resin. The purified protein was then conjugated with verteporfin. The procedure involved extracting verteporfin from liposomal Visudyne (QLT Inc.) by acidification with 6 M HCl and separation of organic and aqueous layers. Verteporfin was activated with EDC (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) followed by incubation with fVII-S-His protein. The conjugate was purified on a spin column (G50 Sephadex; Roche Diagnostics, Indianapolis, IN), and the concentrations of protein and verteporfin were determined.

Fluorescein Angiography
The rats were anesthetized and their pupils dilated with 2.5% phenylephrine and 1.0% tropicamide. Fluorescein angiography was performed on sections every 12 cuts. The sections with lesions were prepared. Hematoxylin-eosin staining for light microscopy was performed on sections through the CNV lesion and the adjacent retina. This image shows the hyperfluorescence at the site of laser injury. Pathologic new vessels in CNV membrane grows from the choroid into the retina. This image shows the hyperfluorescence at the site of laser injury. (B) The late phase of the fluorescein angiogram of the same eye 7 weeks after laser photocoagulation, showing the presence of leakage of CNV.

Table 1. Experiment Design

<table>
<thead>
<tr>
<th>Day</th>
<th>Time of Laser Irradiation Following Intravenous Injection (min)</th>
<th>Treated Lesions (n)</th>
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<tr>
<td>0.5</td>
<td>30</td>
<td>36</td>
</tr>
<tr>
<td>1.0</td>
<td>60</td>
<td>7</td>
</tr>
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<td></td>
<td>30</td>
<td>53</td>
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Histopathologic and Immunohistochemical Study
Twelve fVII-conjugated verteporfin TPT-treated eyes were prepared for hematoxylin-eosin staining and for immunohistochemical staining for vascular endothelial marker CD31 on days 1, 3, and 7 after treatment. Similar histopathologic examination was performed on six untreated eyes as the control. The rats were euthanatized by CO2 asphyxiation, and the globes were carefully enucleated, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), and embedded in paraffin. Each eye was cut through the optic nerve and 4-μm-thick sections were prepared. Hematoxylin-eosin staining for light microscopy was performed on sections every 12 cuts. The sections with lesions were deparaffinized with xylene followed by rehydration with graded dilutions of ethanol, washing in PBS, and incubation in 0.3% H2O2 in 4°C for 15 minutes. The sections were then incubated in 10% normal goat serum for 1 hour in 37°C. Slides were stained separately with a rabbit polyclonal antibody against CD31 (PECAM-1; WM-59; Santa Cruz Bio-
technology, Santa Cruz, CA) for endothelial cells, then reacted with a secondary antibody conjugated with alkaline phosphatase (Vector Laboratories Inc, Burlingame, CA). They were washed with PBS, stained with a red substrate system (Vector Laboratories), and counterstained with hematoxylin. They were then irrigated with tap water, dried, mounted with anti-fade mounting, coverslipped, and placed under a microscope for observation.

**Figure 2.** Standard nontargeted verteporfin PDT for CNV in a rat model. (A) Early and (B) late phases of fluorescein angiogram of Brown-Norway rat 3 weeks after argon green laser induction of CNV. Six CNV (C1–C6) spots were seen with leakage on early phase and late hyperfluorescence. (C) Early phase 24 hours after intravenous verteporfin PDT with 6.0 mg/m² verteporfin and fluence of 50 J/cm². C1, C3, and C5 were treated with 689 nm laser 15 minutes after verteporfin injection. Untreated lesions (C2, C4, and C6) had leakage as before. (D) The late phase of the same fluorescein angiogram as shown in (C). All treated lesions (C1, C3, and C5) showed hyperfluorescence, whereas the leakage of the three untreated spots (C2, C4, and C6) was similar to before treatment (B). (E) Late phase 7 days after verteporfin PDT. All treated (C1, C3, and C5) and untreated lesions showed hyperfluorescence in the late phase. (F) Late phase 14 days after verteporfin PDT. All lesions, including treated CNVs (C1, C3, and C5), showed leakage.

**Table 3.** Closure Rate of CNV 7 and 14 Days after Treatment with Standard PDT or TPT

<table>
<thead>
<tr>
<th>Laser-Activated Agent for Intravenous Injection</th>
<th>Intravenous Dose (mg/m²)</th>
<th>Time of Laser Irradiation Following Intravenous Injection (min)</th>
<th>Efficacy of Treatment in Stopping Leakage of Vessels in the CNV</th>
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</thead>
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<tr>
<td>Liposomal verteporfin</td>
<td>6.0</td>
<td>15</td>
<td>25% (3/12)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>30</td>
<td>0% (0/12)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>60</td>
<td>67% (24/36)</td>
</tr>
<tr>
<td>iVII-conjugated verteporfin</td>
<td>6.0</td>
<td>30</td>
<td>43% (5/7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>75% (40/53)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>30</td>
<td>62% (15/21)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>19% (4/21)</td>
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The number of CNV lesions in each group is presented in parentheses.
viewed with a microscope (Axioplan 2; Carl Zeiss Meditec, Oberkochen, Germany).

RESULTS

Fluorescein Angiography of Untreated CNV

Fluorescein angiography showed the vascular network of the retina and choroid. Argon green laser through a slit lamp adaptor was used to induce CNV. New vessels grew and formed a choroidal neovascular membrane. The fluorescein angiography of untreated CNV showed early hyperfluorescence with late phase leakage at the site of laser injury 7 days after laser photocoagulation and reached its peak on about day 21 (Fig. 1A). The hyperfluorescence of CNV was still present 7 weeks after laser induction, indicating that laser-induced CNV does not regress spontaneously for at least 7 weeks.

Nontargeted PDT with Verteporfin

Three Brown-Norway rats were treated with nontargeted PDT. After regular intravenous injection of 6.0 mg/m² liposomal verteporfin and standard PDT, angiographic closure of experimental CNV was evaluated at 24 hours and 3, 7, and 14 days. After 24 hours there was a circle of choroidal hypofluorescence in the early phase of fluorescein angiography that corresponded to the irradiation area, but the area was smaller than for standard PDT. Untreated lesions (C₁₅) continued to show leakage. (D) Late phase of the same eye shown in (C). All the treated CNV spots (C₁₂₅) showed hyperfluorescence corresponding to the 689 nm laser-treated area; the leakage of the other two untreated spots (C₃₄) remained the same as before treatment. (E) Late phase 7 days after TPT. Two treated spots (C₁₂₅) show closure of the CNV with no leakage of fluorescein, but just light staining in the late phase, and one spot has mild late leakage (C₅). (F) Late phase of fluorescein angiogram 14 days after verteporfin TPT. CNVs in all treated lesions were closed (C₁₂₅), showing light staining and no leakage in the late phase, whereas the untreated lesions (C₃₄) continued to show leakage.
TPT with fVII-Conjugated Verteporfin

In present study, two doses of fVII-conjugated verteporfin were studied: 0.5 and 1.0 mg/m². A 689-nm PDT laser was applied for 30 minutes (group 1) or 60 minutes (group 2) after IV injection. In a pilot study, several other doses and timing were studied, and then the optimal treatment factors for the present study were determined. The efficacy of TPT was evaluated by fluorescein angiography after 1, 3, 7, and 14 days. On day 1, all treated lesions showed hypofluorescence in the early phase, followed by hyperfluorescence in the late phase corresponding to the area of 689-nm laser irradiation. Seven-day closure rates of CNVs treated 30 minutes after injections of 0.5 and 1.0 mg/m² fVII-conjugated verteporfin were 67% (24/36) and 75% (40/53), respectively ($P = 0.36$). The closed CNV showed hypofluorescence in the treated area in the early phase of fluorescein angiography and a light staining of the lesion in the late phase (i.e., lesion C in Fig. 3F). Fourteen-day closure rates of 0.5 and 1.0 mg/m² were 64% (23/36) and 77% (41/53), respectively ($P = 0.17$). On day 14, the closure rate of lesions treated 30 minutes after injection of fVII-conjugated verteporfin were higher than those that were treated 60 minutes after injection in both dosage groups (Table 3, Fig. 3).

No ocular side effects, vascular contraction, or scar formation were detected after TPT. There was less blanching of normal choroidal vessels around CNV lesions in TPT-treated CNVs compared with standard PDT in the early phase of fluorescein angiography 24 hours after treatment.

**Histopathologic and Immunohistochemical Analyses**

In all the control eyes that had laser-induced CNV but were not treated, choroidal neovascular membranes were found with patent new vessels and hemosiderin-laden macrophages by hematoxylin-eosin staining under light microscopy (Fig. 4A). The choroidal neovascular membrane progressed over time to become more fibrotic. The membrane was hypocellular with limited inflammatory response. Figure 4B shows a light microscopic image of an untreated choroidal neovascular membrane 3 weeks after induction with argon green laser (PECAM-1 staining). The CNV contained a mixture of diffused patent

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**Figure 4.** Immunohistochemical staining of CNV in rat eyes. (A) Hematoxylin-eosin staining of an untreated CNV membrane; black arrows: the new vessels in the CNV membrane. (B) PECAM-1 staining of the specimen shown in (A), counterstained with hematoxylin. The neovascular membrane shows fibrosis, melanin pigment granules, and thin-walled vessels with patent lumens. (C) Hematoxylin-eosin staining of a CNV membrane 24 hours after intravenous TPT. (D) The same lesion as shown in (C), PECAM-1-stained CNV membrane. CD31 was highly expressed in CNV lesion and ocular tissues around treated CNV. (E) PECAM-1 staining of CNV 7 days after intravenous TPT. New vessels are not identifiable, suggesting that they have either regressed or closed. V, vitreous; R, retina; S, sclera; Ch, choroid.
vascular structures with red blood cells in the cavity. The vessels were prominent and patent 24 hours after TPT (Fig. 4D). The new vascular channels regressed and closed 7 days after TPT (Fig. 4E).

**DISCUSSION**

Visudyne (QLT, Inc.), a liposomal formulation of verteporfin, has been shown to be effective for the treatment of CNV in selected AMD patients.1,2 Visudyne-mediated PDT affects not only the CNV but also causes hypoperfusion of normal choroidal vessels. More efficacious and safer procedures for PDT are needed to improve the outcome of PDT. Our procedure involves conjugating verteporfin to fVII, a ligand that binds with exceptional affinity and specificity to its receptor TF, which is expressed selectively on endothelial cells of CNV. The fVII-verteporfin conjugate targets verteporfin to the CNV, thus avoiding side effects from nonspecific binding of verteporfin to the normal vasculature. A similar agent, fVII/hIgG1 Fc (Icon), was injected intravitreally and intravenously in our previous studies in mouse and pig models of CNV.11,12 It was shown that both mouse and human Icon (fVII/hIgG1 Fc) proteins could bind to neovascular choriocapillaris endothelial cells but not normal endothelium (Figs. 1–3 in Ref. 11 and Fig. 2 in Ref. 12), and destruction of CNV was observed by confocal microscopic imaging as a result of the binding of Icon to the CNV (Fig. 4 in Ref. 11 and Fig. 5 in Ref. 12). Since CNV endothelial cells were immunostained with Icon in frozen sections, the findings about specific binding of fVII to CNV in the previous studies could be applied to the present study.

The route of administration of 10% sodium fluorescein was intraperitoneal injection. We started imaging right after the injection. Fluorescein was usually detected in retina arteries 3 to 15 seconds after injection. Early was defined as the first 60 seconds after intraperitoneal injection of fluorescein. Late was defined as more than 5 minutes after the injection of fluorescein. We tried to compare fluorescein images with similar timing to reduce bias. Moreover, the corresponding histopathologic examination 24 hours after either PDT (not shown in the article) or nontargeted PDT (Fig. 4D) also showed a high expression of CD31. This finding seems to be related to injury to CNV due to either targeted or nontargeted PDT. However, this phenomenon disappeared on day 7 after treatment, indicating that the high expression of CD31 was temporary and also that the closure of new vessels after targeted PDT was significant on day 7.

We compared the efficacy and safety of targeted PDT using fVII-verteporfin with nontargeted PDT using Visudyne, in a rat model of laser-induced CNV. The assay involved detecting leakage in blood vessels of the CNV 21 days after laser-induced damage through fluorescein angiography. In the control rats that were not treated by PDT and in the rats treated by nontargeted PDT with a dose of 6.0 mg/m² Visudyne, leakage continued in all the vessels. In contrast to the failure of nontargeted PDT to stop leakage, TPT with fVII-verteporfin stopped leakage in 65% of the CNV blood vessels at a dose of 0.5 mg/m² and in 75% of the CNV blood vessels at a dose of 1.0 mg/m² (Fig. 3E, C3 and C4). There was no evidence of side effects involving vascular contraction or scar formation. We conclude that targeted photodynamic therapy using fVII-verteporfin is more efficacious and safer than nontargeted photodynamic therapy using Visudyne.

Another study involving the rat model of laser-induced CNV showed that targeted photodynamic therapy with verteporfin conjugated to a peptide that binds to VEGFR-2 stopped leakage in all the laser-damaged CNV blood vessels on day 1 after PDT, without causing significant damage to the retinal pigment epithelium or photoreceptors.15 This experiment provided evidence that ligand-targeted verteporfin photodynamic therapy could improve the efficacy and safety of PDT for treating the CNV.

The early phase of fluorescein angiography 24 hours after TPT showed only a very mild choroidal hypofluorescence at the site of treated CNV (Fig. 3C). However, there was a significant choroidal hypofluorescence at the treated spot in the early phase of fluorescein angiography 24 hours after standard PDT, which corresponded to nonperfusion of normal choroidal circulation (Fig. 2C). Another study reported similar findings after PDT in monkey eyes.3 Based on fundus photos, fluorescein angiogram and histopathologic evaluation, targeted PDT seems to be safe. No abnormality was observed morphologically in the organs and tissues of the rats during the experiments and/or at the time of death.

In summary, the present experiments reported in a rat model of CNV showed that the leakage of CNV vessels could be treated more efficaciously and safely by using fVII-verteporfin for targeted PDT instead of Visudyne for nontargeted PDT. If further experiments verify these results, laser-activated dyes with fVII for targeted PDT could lead to improvement in the treatment of AMD.

**Acknowledgments**

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**References**


