Synergies of VEGF Inhibition and Photodynamic Therapy in the Treatment of Age-Related Macular Degeneration

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PURPOSE. Photodynamic therapy (PDT) and the administration of compounds acting against vascular endothelial growth factor (anti-VEGF) are approved for the treatment of choroidal neovascularization (CNV) secondary to age-related macular degeneration (AMD). Experimental evidence that the combined use of both treatment options may improve therapeutic outcome is presented.

METHODS. Fertilized chick eggs were incubated until day 12 of embryo development (EDD12) and were treated by PDT using two different photosensitizing agents (liposomal formulation of BPD-MA; m-THPP encapsulated in polymeric nanoparticles) and were visualized using an epifluorescence microscope. Vascular occlusion of the treated zones of the chorioallantoic membrane (CAM) was assessed by fluorescence angiography 24 and 48 hours after alternative treatment. PDT-treated areas were exposed to a soluble VEGF receptor antagonist (sFlt-1) 6 hours after treatment and were analyzed.

RESULTS. Vascular occlusion in the PDT-treated areas was observed with both photosensitizers 24 hours after treatment. Reperfusion of preexisting blood vessels and first signs of revascularization were visible 48 hours after PDT. Topical administration of sFlt-1 to the treated areas augmented occlusion and limited subsequent angiogenesis in a dose-dependent manner.

CONCLUSIONS. The combined use of PDT and of agents targeting angiogenic cytokines may synergistically improve therapeutic outcome after combined treatment in patients with CNV secondary to AMD. (Invest Ophthalmol Vis Sci. 2007;48:1767–1772) DOI:10.1167/iovs.06-1224

The wet form of age-related macular degeneration (AMD) can be treated by three proven therapies.1 Although thermal laser treatment is still used for the nonspecific coagulation of choroidal neovascularization (CNV) at extrafoveal sites,1 more directed agents include photodynamic therapy (PDT) and anti-VEGF therapy. These agents provide benefit through more specific inhibition of disease-associated neovascularization with minimal damage to retinal vessels, normal choriocapillaries, retinal pigment epithelium, and adjacent photoreceptors.

In liposomal benzoporphyrin derivative monoacid ring A–mediated PDT, the photosensitizer BPD-MA is applied intravenously, followed by irradiation of the diseased site with 689-nm light. Activation of the photosensitizer then leads to the local production of reactive oxygen species that trigger vascular occlusion through a complex cascade of molecular, cellular, and physiological events.2 Because of the predominantly vascular localization of the photosensitizer directly after administration, collateral damage to neighboring tissues is not extensive. This treatment modality, approved for predominantly classic wet AMD, has been shown in several clinical trials to stabilize or slow vision loss.3–7

Because the expression of angiogenic stimuli, such as vascular endothelial growth factors (VEGFs), basic fibroblast growth factors (bFGFs), and the angiopoietins, is involved in the pathogenesis of CNV associated with AMD,8–11 another option to treat this disease is the local administration of agents that interfere with the activity of these cytokines. VEGF is a major stimulator of angiogenesis.12 Consequently, two anti-VEGF agents, pegaptanib sodium and ranibizumab, have recently demonstrated convincing results in pivotal phase 3 studies. While the latter is a humanized antibody fragment that binds to all VEGF isoforms, pegaptanib sodium is a pegylated 28-base RNA oligonucleotide that binds specifically to the heparin-binding VEGF-A isoform. Both agents are administered intravitreally with minimal complications.

In PDT, microvascular injury induces inflammation and hypoxia and the expression of angiogenic and survival molecules including VEGFA, which could lead to CNV persistence and recurrence. For that reason, it is likely that multiple rounds of AMD retreatment are necessary.13 Because PDT and anti-VEGF therapy act on CNV according to different mechanisms, their combinational use might represent a benefit for patients undergoing treatment of AMD. This combination therapy is extremely promising, particularly if VEGF-A–inhibiting agents are used after PDT, because PDT is able to remove unwanted CNV whereas anti-VEGF therapy will alter the progression of the disease and maintain the PDT effect.

To elucidate PDT/anti-VEGF combination therapy, we studied the angiogenic response after PDT in the chick chorioallantoic membrane (CAM) model with two different photosensitizers and the influence of an anti-VEGF agent on CAM vascularization after PDT. Liposomal-formulated BPD-MA was used as a reference photosensitizer for the treatment of CNV and mTHPP encapsulated in nanoparticles to elucidate whether formulation, pharmacokinetics, or photochemical properties have an influence on the potential synergistic effect.
Materials and Methods

Materials

The following materials were used: recombinant human soluble VEGF R1 (sFlt-1)/Fc chimera (R&D Systems Europe Ltd., Abingdon, Oxford, UK); liposomal benzoporphyrin derivative monocoid ring A (BPD-MA; Visudyne; Novartis, Basel, Switzerland); poly(D,L-lactide-co-glycolide) (PLGA) with a copolymer ratio of 50:50 and a molecular weight of 12 kDa (Resomer; RG502; Boehringer Ingelheim, Ingelheim, Germany); meso-tetra (p-hydroxyphenyl)porphyrin (m-THPP; Aldrich, Steinheim, Germany); poly(vinyl alcohol) 87.7% hydrolyzed with a molecular weight of 26 kDa (Mowiol 4 to 88; Hoechst, Frankfurt/Main, Germany); D(+)-Trehalose dihydrate and phosphate-buffered saline (PBS; Sigma, Aldrich, Taufkirchen, Germany); sulforhodamine 101 and ethanol 99.8 (Fluka, Buchs, Switzerland); Lipidm 10% (Vifor SA, Geneva, Switzerland). All chemicals were of analytical grade and were used without further purification.

Microscope Setup

Fluorescence imaging of CAM vessels was performed with a Retiga EX camera (QImaging, Burnaby, BC, Canada) fitted to a fluorescence microscope (Eclipse 600 FN; Nikon, Tokyo, Japan) equipped with an objective CFI achromat magnification of 4×, a numerical aperture of 0.10, and a working distance of 30 mm. Illumination was provided by a filtered 100-W mercury arc lamp. Light doses were measured with a calibrated FieldMaster power meter (Coherent, Santa Clara, CA). For the studies with m-THPP, the microscope was equipped with a fluorescence cube (BV-2A; Nikon). This cube is composed of a 420 CWL filter, which provides excitation wavelengths between 400 and 440 nm, a dichroic mirror (455 nm), and a long path filter (470 nm). An additional band path filter (D650/50m; Chroma Technology Corp., Rockingham, VT) was added. For the studies with BPD-MA, a fluorescence cube composed of a 420 CWL filter, a dichroic mirror (455 nm), and a long path filter (610 nm) was assembled. The fluorescence of sulforhodamine 101 was detected with a cube (G-2B; Nikon) composed of a D535/50× excitation filter, a dichroic mirror (575 nm), and a long path filter (610 nm). An additional band path filter (D650/50m; Chroma Technology Corp.) was added. Digital imaging, data display, and storage were performed with commercial hardware (Macintosh; Apple, Cupertino, CA) connected to the CCD camera and the software (OpenLab 3.15; Improvision Ltd., Coventry, UK).

Nanoparticle Preparation

Nanoparticles loaded with m-THPP were prepared using the emulsification-diffusion technique, as described by Vargas et al. The mean diameter of the freeze-dried nanoparticles, determined by photon correlation spectroscopy (Zetasizer 5000; Malvern, Worcestershire, UK), was 125 ± 13 nm with a polydispersity index of 0.2. A photon-sensitizer loading of 7.3% (wt/wt) was determined spectrophotometrically with a spectrometer (Cintra 40; GBS, Victoria, Australia) using a calibration curve established at 688 nm.

Egg Incubation

Fertilized hen eggs (Animalerie universitaire; University of Geneva, Geneva, Switzerland) were placed into an incubator (MG 200; Savimat, Chauffry, France) set at 37°C and a relative humidity (RH) of 65%. Until EDD 4, eggs were automatically rotated. A 3-mm hole was drilled into the eggshell at the narrow apex and was covered with an adhesive tape. Eggs were then incubated without rotation until the CAM assay on EDD 12.

Chick Chorioallantoic Membrane Preparation and Injection Procedure

The CAM assay was adapted from Lange et al. with minor modifications. All assays were performed at least 5 times, unless otherwise specified. On EDD 12, the hole in the eggshell was enlarged to a diameter of 2 to 3 cm, allowing access to the CAM vasculature. Chick embryos were placed under the objective of the fluorescence microscope. For m-THPP doses of 0.6 and 1.2 mg/kg body weight of chick embryo in PBS and for BPD-MA, 0.20 and 0.25 mg/kg doses in water were used. Formulations were injected into one of the principal blood vessels of the CAM through a 33-gauge needle fitted to a 100-μL syringe (Hamilton, Reno, NV).

Photodynamic Therapy and Assessment of Vascular Occlusion

CAM was irradiated with light doses of 15, 25, or 30 J/cm² at 400 to 440 nm 1 minute after IV administration of photosensitizer. The surface of the irradiated CAM area was 1.8 mm². Before PDT, a silicone O-ring (Apple Rubber Products Inc., Lancaster, NY) was placed to frame the region on which PDT was performed. Subsequently, the aperture in the shell was carefully covered with a plastic film (Parafilm; Pechiney Plastic Packaging, Chicago, IL), and treated eggs were maintained in the dark for 24 hours in the incubator (37°C, 65% RH). Then, 20 μL sulforhodamine 101 in NaCl 0.9% (0.5 mg/mL) was injected into chick embryos to document vascular occlusion after PDT. The same procedure was carried out 48 hours after PDT to determine the potential neovascularization of the treated area. To improve visualization of the vascular bed at 48 hours, the contrast medium (Lipidm 10%; Vifor SA) was injected under the chorioallantoic membrane. Comparison of vessel fluorescence before and after PDT (24 and 48 hours) allowed an evaluation of vessel occlusion using an arbitrary damage scale proposed by Lange et al. presented in Table 1.

Anti-VEGF Therapy

After the hole in the eggshell was enlarged to a diameter of 2 to 3 cm on EDD 12, a silicone O-ring was placed on the CAM, the aperture in the shell was carefully covered with a plastic film, and the eggs were maintained for 6 hours in the incubator (37°C, 65% RH). Next, 10 μL sFlt-1 solution in PBS (0.50, 0.75, 1.00, or 2.00 μg/embryo) was topically applied inside the O-ring, and the shell was covered again with a plastic film. After 24 and 48 hours, fluorescence angiography of the irradiated area with sulforhodamine 101 was performed, as described.

Table 1. Evaluation of PDT-Induced Damage on CAM Vessels

<table>
<thead>
<tr>
<th>Damage Scale*</th>
<th>Criterion</th>
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<tbody>
<tr>
<td>0</td>
<td>No damage</td>
</tr>
<tr>
<td>1</td>
<td>Partial closure of capillaries of diameter &lt;10 μm</td>
</tr>
<tr>
<td>2</td>
<td>Closure of capillary system, partial closure of blood vessels of diameter &lt;30 μm, and size reduction of larger blood vessels</td>
</tr>
<tr>
<td>3</td>
<td>Closure of vessels of diameter &lt;30 μm and partial closure of higher order vessels</td>
</tr>
<tr>
<td>4</td>
<td>Total closure of vessels of diameter &lt;70 μm and partial closure of larger vessels</td>
</tr>
<tr>
<td>5</td>
<td>Total occlusion of vessels in the irradiated area</td>
</tr>
</tbody>
</table>

* Intermediate values were found when different scores were averaged.
Photodynamic Therapy and Anti-VEGF Therapy Synergies and Damage Assessment

After photosensitizer injection and irradiation under the conditions mentioned, treated eggs were maintained in the dark for 6 hours in an incubator (37°C, 65% RH). sFlt-1 solution (10 μL) was topically applied, and fluorescence angiography of the irradiated area with sulforhodamine 101 was performed 24 and 48 hours after PDT. Comparison of vessel fluorescence before and after PDT and both PDT and VEGF treatments allowed an evaluation of vessel damage using the same arbitrary damage scale.

Statistical Analysis

Nonparametric analysis was performed to compare the effect of single-treatment PDT and anti-VEGF and combined-treatment PDT/anti-VEGF by means of the Kruskal-Wallis or the Jonckheere test, followed by post hoc multiple comparison.

RESULTS

Photodynamic Therapy and Damage Assessment

The CAM model used to study AMD consists of controlling the CAM vasculature before, during, and 24 hours after the desired therapy. However, to assess undesirable effects such as reperfusion and repeat neovascularization in PDT, the CAM vasculature was also assessed 48 hours after therapy. The effect of PDT after 24 hours and 48 hours with m-THPP-loaded nanoparticles (drug dose, 0.60 and 1.20 mg/kg embryo) and BPD-MA liposomes (drug dose, 0.20 and 0.25 mg/kg) on the vascular system of the CAM using 30 J/cm² as light dose are summarized in Figure 1.

All conditions resulted in dose-dependent vascular occlusion after 24 hours. However, reperfusion of occluded vessels after 48 hours led to a considerable decrease in damage score. Formation of new vessels was particularly evident at 30 J/cm² (data not shown for other light doses). Optimal conditions to study angiogenesis activity were achieved using drug doses of m-THPP 1.2 mg/kg embryo (Fig 1A) and BPD-MA 0.25 mg/kg embryo (Fig 1B), respectively. These conditions resulted in strong vascular occlusion 24 hours after PDT and in neovascularization and reperfusion of larger vessels (>70 μm) after 48 hours.

Figure 2 shows the typical sequence of a CAM assay. It consisted of injection of a photosensitizer (Fig. 2A), followed by PDT (Fig. 2B) and sulforhodamine 101 angiography 24 hours after PDT (Fig. 2C). In addition to the standard sequence, sulforhodamine 101 angiography with a contrast medium was again performed, this time 48 hours after PDT (Fig. 2D). With the use of m-THPP under the conditions described here, total closure of the capillary system and blood vessels with diameters smaller than 30 μm and size reduction of larger blood vessels were observed after 24 hours (Fig. 2C). However, 48 hours after PDT, previously reduced vessels regained their original sizes, and some vessels that appeared to be occluded were reperfused (Fig. 2D). Furthermore, the entire region was recovered by newly formed, smaller blood vessels (diameter, <20 μm) that were morphologically different from those outside the treated areas. Similarly, high vascular occlusion 24 hours after PDT was found for BPD-MA (Fig. 2IC), as was the regrowth of previous vasculature and the formation of new morphologically different vessels after 48 hours (Fig. 2ID). Therefore, regardless of the photosensitizer, signs of vascular reperfusion and neoangiogenesis could be observed 48 hours after PDT in the CAM.

Anti-VEGF Therapy

Anti-VEGF-induced changes were followed 24 hours and 48 hours after topical administration by fluorescence angiography. Autofluorescence of vessels of the CAM area exposed to drug was also recorded before administration for comparison. Figure 3 illustrates the closure of blood vessels to different extents with varying doses of sFlt-1.

Increased damage was exhibited with increasing doses of sFlt-1. However, doses greater than 1 μg/embryo frequently led to changes of vascular architecture morphology and partial destruction of the CAM.

Synergies of Photodynamic Therapy and Anti-VEGF Therapy

Results of PDT with BPD-MA in combination with increasing doses of sFlt-1 are summarized in Figure 4. Figure 4A clearly illustrates the differences between BPD-MA-PDT therapy (Fig 2II) and BPD-MA-PDT/sFlt-1 combined therapy (Fig 4A). Representative damage 24 hours after PDT was observed with BPD-MA (drug dose, 0.25 mg/embryo; light dose, 30 J/cm²), followed by the reperfusion of larger vessels and the generation of new vessels 48 hours after PDT (Figs. 2IC, 2ID, respectively). However, vascular occlusion induced by the combined therapy (PDT, same conditions; sFlt-1, 1 μg/embryo) 24 hours after PDT (Fig. 4A) was higher than for PDT alone (Fig. 2IC). Furthermore, after 48 hours, combined treatment resulted in only minimal reperfusion in the PDT-treated area.

A statistically significant increase (P < 0.05) of vascular occlusion was induced by the use of sFlt-1 in combination with PDT after 24 hours (Fig 4B), from 3.4 U (PDT alone) to approximately 4.6 U (combined PDT/anti-VEGF). This increase represented the occlusion not only of capillaries and of vessels smaller than 70-μm diameter but also of vessels larger than 70-μm diameter. Furthermore, vascular reperfusion and neovascularization 48 hours after treatment was significantly re-
duced ($P < 0.01$). Optimal results were obtained with the BPD-MA-PDT/sFlt-1 1 μg/embryo, leading to an average damage of 3.8 after 48 hours. This resulted in more extensive vascular injury than that observed after PDT alone (1.4). Moreover, 48 hours after PDT, no statistically significant damage was observed compared with the effect observed after 24 hours (4.6) with combined therapy ($P > 0.01$), indicating stabilization of the treatment outcome. Compared with sFlt-1 therapy alone, combined therapy PDT/sFlt-1 resulted in a highly significant increase in vascular damage ($P < 0.01$) at 48 hours, with a reduction of more than one third of the vascular reperfusion and neovascularization. No differences between PDT and s-Flt-1 as individual therapies could be established at 24 or 48 hours ($P > 0.01$; see Fig. 3).

**DISCUSSION**

We have previously shown that the CAM model can be efficiently used to screen different parameters including photosensitizer, light dose, and dose-light interval, for optimized PDT of CNV. In the present study, we have extended the potential use of this simple, yet effective, model to two additional aspects: the observation of angiogenic activity after PDT and the investigation of synergetic effect anti-VEGF treatment and PDT.

We have shown that liposomal BPD-MA or nanoencapsulated m-THPP, vascular reperfusion of previously occluded

![Figure 2. Assessment of vascular occlusion after photodynamic therapy. Fluorescence angiographies of PDT on CAM vasculature using intravenously applied (I) m-THPP nanoparticles (1.20 mg/kg embryo), (II) BPD-MA (0.25 mg/kg embryo). (A) Photosensitizer fluorescence angiography before PDT. (B) Photosensitizer fluorescence angiography during PDT. Diameter of the irradiated area, 1.8 mm; irradiation condition, 30 J/cm² 1 minute after photosensitizer injection. (C) Sulforhodamine 101 fluorescence angiography 24 hours after PDT.](image)

![Figure 3. Vascular occlusion induced by sFlt-1. Vascular occlusion induced by sFlt-1 in the CAM as a function of anti-VEGF concentration 24 hours (●) and 48 hours (□) after topical application. Mean ± SD (n = 5).](image)

![Figure 4. Synergistic effects from the combined use of Photodynamic therapy and anti-VEGF agents. (A) Sequence of fluorescence angiographies (see Fig. 2) with BPD-MA (0.25 mg/kg embryo). PDT followed by anti-VEGF therapy using topically applied sFlt-1 (1 μg/embryo) 6 hours after PDT. Damage score after 24 hours: BPD-MA-PDT/sFlt-1, 5. Damage score after 48 hours: BPD-MA-PDT/sFlt-1, 4. White bar: 500 μm. (B) Vascular damage induced by the sFlt-1 topically applied 6 hours after BPD-MA-PDT (0.25 mg/kg; light dose, 30 J/cm²) and 24 hours (■) and 48 hours (□) after PDT. The first concentration (0.00 μg/embryo) represents PDT alone.](image)
blood vessels, and formation of new blood vessels exclusively in the treated areas can be observed independently of the photosensitizer or formulation used. Therefore, this response is presumably intrinsic to this treatment modality.

It has already been shown that cytokines such as VEGF, tumor necrosis factor-α, and PEGF are expressed after PDT in patients with tumors16–18 and CNV associated with AMD.19,20 Furthermore, it has been shown in tumor-bearing mice that the coadministration of antiangiogenic compounds after PDT is beneficial with respect to disease progression and survival.21

Others have shown that the activation of neutrophils and macrophages induced by PDT can be beneficial with respect to the therapeutic outcome in patients with cancer.22–25 However, because this scenario and the subsequent onset of inflammation is unfavorable with respect to the treatment of CNV, the administration of corticosteroidlike molecules after PDT is recommended for patients with AMD.13,24,25

On a purely vascular level, PDT has been shown to induce damage, namely through alterations in endothelial cells and in the basement membrane. After the establishment of thrombogenic sites in the vascular space, platelet aggregation, release of vasoactive compounds, leukocyte adhesion, increased vascular permeability, and vessel obstruction have been observed in response to PDT. These effects lead to vascular obstruction and collapse, resulting in a hypoxic environment. Results of the present study, however, revealed that the vascular obstruction was partially reversible 48 hours after PDT, in agreement with the characteristic hypofluorescence observed in treated areas of patients 1 week after PDT fluorescence angiography, which resolves relatively rapidly. This rapid resolution indicates the reperfusion of most vascular lumina in those areas.

Tissue hypoxia induced by the occlusion of CNV or smaller choriocapillaries after PDT, however, triggers associated gene activation of the hypoxia-inducible factor 1α (HIF-1) transcription factor complex.26–27 Under normoxic conditions HIF-1α is rapidly degraded, but oxygen deprivation results in the stabilization of the HIF-1α subunit of the heterodimeric HIF-1 active protein complex, leading to the activation of genes involved in the transcription of cytokines including VEGF-A, erythropoietin, and glucose transporter-1.28

Immunohistopathologic examination of eyes of patients who underwent enucleation after PDT clearly revealed the elevated expression of VEGF-A. Furthermore, a correlation among the upregulation of VEGFR-3, the staining for pigment epithelium-derived factor, and the area of treatment was found.29 These findings, together with the results of the present study, might explain clinical observations after AMD-PDT, including hypofluorescence, continuous recurrence, vascular leakage, and progressive loss of visual acuity after multiple PDT sessions.

Conversely, the present study has clearly shown a benefit of the coadministration of compounds acting against VEGF-A because exposure of PDT-treated areas to sFlt-1 after PDT limited the formation of newly formed blood vessels in our model. This is more surprising if one considers that antiangiogenic support was useful even in such a purely neovascular model that did not include as much surrounding tissue, damage, and inflammation as found in patients with AMD.

VEGF-A exists in four major biological isoforms active in humans and containing 121, 165, 189, and 208 amino acids. Animal model data suggest that the 165-amino acid isoform (VEGF165) is primarily responsible for the pathogenesis in ocular neovascularization.30,51 sFlt-1 has been reported to bind this isoform, preventing interaction with VEGFR-1 (Flt-1); fms-like tyrosine kinase and VEGFR-2 (KDR; kinase insert domain-containing region).52,53 Although human isoforms have been studied extensively, little is known about chick counterparts. It has been shown that exposure of the CAM to different angiogenic stimuli, including VEGF and bFGF, resulted in increased angiogenic activity in this model.34 In these studies, VEGF was less potent in inducing angiogenesis than bFGF. It is also known that soluble Flt-1 binds chicken VEGF.35

In our study, optimal dosing was not determined, and relatively high doses of sFlt-1 were applied to produce an observable effect and to show the benefits of using PDT in combination with clinically available drugs. Another factor that should be investigated more thoroughly is the optimal time point of administration of anti-VEGF agents. We have clearly revealed a synergistic effect with the combined use of both treatment options for CNV associated with AMD. However, if our data can be extrapolated to the clinical use of a combination therapy, care should be taken to optimize dosing to protect larger vessels from occlusion.

**References**


