Ocular Changes after Photodynamic Therapy

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PURPOSE. The aim of this study was to identify the changes in the primate visual system after a single session of photodynamic therapy (PDT) in an intact nonhuman primate retina.

METHODS. As part of a larger study, PDT (wavelength 689 nm, 50 J/cm², 600 mW/cm², 83 seconds, 4-mm spot size) with verteporfin (6 mg/m² intravenous infusion) was performed in one eye each of two cynomolgus monkeys. Fundus photography, fluorescein angiography (FA), indocyanine green angiography (ICG), optical coherence tomography (OCT), and multifocal electroretinography (mERG) were performed at baseline and 12 time points (1–283 days) after PDT. In addition, retinal histopathologic findings were evaluated at 9 months.

RESULTS. Various morphologic changes, including whitening of the treated area, RPE proliferation, closure of the choroidal vasculature, and subretinal edema (followed by foveolar thinning) were observed. Most of the changes persisted and were detectable in histopathologic evaluation at 9 months. Reductions of the mERG amplitude, followed by varying degrees of recovery from the treated and the border regions, were observed. This was accompanied by progressive delay of P1 peak time up to 3 months after treatment, followed by complete recovery at 9 months. In addition, the nontreated area showed amplitude and timing mERG deficits, which underwent gradual (but not complete) recovery.

CONCLUSIONS. In a primate model, under standard clinical parameters, a single PDT treatment resulted in various dynamic morphologic and functional retinal changes detectable for up to 9 months after treatment. The significance of the observed changes and possible ways of pharmacologic interference with PDT adverse effects are discussed. (Invest Ophthalmol Vis Sci. 2006;47:377–385) DOI:10.1167/iovs.05-0838

Photodynamic therapy (PDT) was developed at the beginning of the 20th century as a form of cancer therapy.1 However, its widespread application in various medical fields started with the introduction of lasers as powerful and localized light sources in the 1970s. In this two-step procedure, a photosensitizing drug is first administered to the patient, usually intravenously. After a delay to allow for accumulation and selective binding, the treatment area is illuminated with low-power laser light corresponding to the absorption peak of the drug.2 It is assumed that minimal damage is present in nonpathologic tissue. In ophthalmology, a circular spot of laser light is directed and focused inside the eye through a slit lamp, it is used to treat tumors and neovascular formations in the iris3 and the retina.4 Recently, it gained popularity as treatment of choroidal complexes, the most frequent of which is choroidal neovascularization (CNV) associated with age-related macular degeneration (ARMD).5–8

Verteporfin (Visudyne; Novartis Pharmaceuticals Corp., East Hanover, NJ) was approved in the United States by the Food and Drug Administration in 2000 and approved in Europe in 2002 for the treatment of predominantly classic subfoveal CNV secondary to ARMD. In addition, the drug is used for the treatment of minimally classic and occult CNV secondary to ARMD, presumed ocular histoplasmosis syndrome9 and CNV associated with pathologic myopia.10 Encouraging results have been published for the treatment of idiopathic subfoveal choroidal neovascular lesions.11 Verteporfin PDT has exhibited a good safety profile and minimal adverse effects.12 Nevertheless, some reports indicate that single and repeated PDT applications could cause limited damage to the intact retina, choroid, and optic nerve, as revealed by morphologic methods in rats,13,14 rabbits,15 monkeys,16,17 and humans.18,19 However, little is known about the long-term morphologic and histopathologic changes (beyond 6 weeks). In addition, the functional consequences on the treated and the neighboring nontreated areas of an intact central retina are unknown.

The purpose of this study was to identify long-term (up to 9 months) functional and morphologic changes in the primate visual system after single application of PDT using verteporfin.

MATERIALS AND METHODS

As part of a larger study, PDT was performed on the left eye each of 2 cynomolgus monkeys, each a 4-year-old female weighing 2.5 to 3 kg, selected based on sex and age match to animals participating in other arms of the study. All tests were performed in accordance with the ARVO guidelines for animal research and were approved by the local institutional review board.

The animals were anesthetized with ketamine (10 mg/kg), paralyzed with vecuronium bromide (30 μg/kg), and maintained on artificial ventilation (with 100% oxygen) during the tests. The pupils were dilated with 1 drop of phenylephrine hydrochloride 2.5% and 1 drop of tropicamide 0.5% to a diameter >6 mm.

Photodynamic Therapy

As part of the protocol for a larger study, 2% dimethyl sulfoxide in H2O, 0.3 mL/kg intravenously (through the saphenous vein) was applied 20 minutes before start of PDT. The dye used during the procedure was verteporfin (Visudyne; Novartis Pharmaceuticals Corp.), administrated at a dose of 6 mg/m² intravenous infusion in 10 mL saline for 10 minutes. A diode laser (OpaL Photoactivator; Lumenis Inc., Santa Clara, CA) coupled with a slit lamp (Carl Zeiss Meditec, Dublin, CA) was used as a light source. Treatment parameters were 689-nm wavelength, 50

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Submitted for publication June 29, 2005; revised August 16, 2005; accepted November 23, 2005.

Disclosure: R. Tzekov, Allergan Inc. (E); T. Lin, Allergan Inc. (E); K.-M. Zhang, Allergan Inc. (E); B. Jackson, Allergan Inc. (E); A. Oyejide, Allergan Inc. (E); W. Orilla, Allergan Inc. (E); A.D. Kulkarni, Allergan Inc. (E); B.D. Kuppermann, Allergan Inc. (C, I); L. Wheeler, Allergan Inc. (E); J. Burke, Allergan Inc. (E).

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J/cm², 600 mW/cm², duration 83 seconds, 4-mm spot size on the retina centered on the fovea (using OGFA-2, NMRK Fundus Laser Lens; Ocular Instruments, Bellevue, WA). Dye-to-laser time was 5 minutes after infusion. The procedure was conducted in a room with reduced illumination to avoid additional light photosensitization.

**Imaging Tests**

A battery of tests was used to evaluate the functional and morphologic status of the retina.

Color and red-free 50° fundus photographs of the posterior pole (centered on the fovea) were obtained with a digitized fundus camera (FF450 IR; Carl Zeiss Meditec). The same camera was used for digital fluorescein angiography according to a standard procedure. Briefly, 1 mL of 5% sodium fluorescein dye was bolus injected intravenously. Photographs were obtained every second for the first 20 seconds and then on 1, 2, 3, 4, 5, and 6 minutes.

Indocyanine green angiography (ICG) was performed with a scanning laser ophthalmoscope (Heidelberg Retina Angiograph; Heidelberg Engineering, Inc., Vista, CA). Red-free and infrared images were captured before injection of 0.5 mL of 5 mg/mL indocyanine green dye (IC-Green; Akorn Inc., Buffalo Grove, IL) administered as an intravenous bolus. At injection, image capture in ICG mode was made using 6 frames per second for the first 30 seconds and then at 1-minute intervals for 5 minutes in the PDT-treated eye. Image resolution was set at 512 × 512 pixels. Choroidal occlusion was scored in a masked fashion by trained graders using a 4-point scale (0 = no effect, 1 = minimal effect, 2 = marginal effect, 3 = marked effect) to compare baseline images to follow-up images of the choroidal phase (approximately 4 to 6 seconds after ICG injection).

Optical coherence tomography (OCT) retina thickness line measurements of the foveal region were obtained with an OCT III system, (Stratus; Carl Zeiss Meditec). Fovea thickness measurements were made using a set of 6 radial lines (6 mm in length). The average of the six lines was determined and presented. In addition, “macular volume” as calculated by the analysis software is presented. Retina thickness analysis tools in the software (OCT III; Carl Zeiss Meditec) were used for analysis.

**Histologic Evaluation**

Eyes were enucleated immediately after the last follow-up visit on day 283. The procedure was performed immediately after euthanatization (Eutha-6; Western Medical Supply Co. Inc., Arcadia, CA). The eyes were fixed in modified Davidson’s fixative overnight. Consecutive sections of posterior pole were cut at 5-µm thickness. Tissue samples were stained with hematoxylin-eosin. Additional consecutive sections were evaluated for glial fibrillary acid protein (GFAP) expression using standard horseradish peroxidase immunohistochemistry and a primary polyclonal anti-GFAP antibody (Dako, Carpinteria, CA).

**Multifocal ERG**

Multifocal ERG (mERG) was performed on anesthetized monkeys (see previous section). Direct fundus observation and projection of the stimulus were obtained with a fundus camera stimulator (Veris 1.5; EDI, San Mateo, CA). mERGs were recorded monocularly with the use of Burian-Allen contact lens electrodes. The stimulation protocol had the following parameters: 241 hexagonal areas, m-sequence at 75-Hz refresh rate, 600 cd/m² stimulus luminance, 98% Michelson contrast, 300 cd/m² background luminance, and exponent m = 13 (resulting in a total recording time of 4 minutes). System software (Veris Science, ver. 4.9; EDI) was used for recording, and a later version (Veris Science, ver. 5.0; EDI) was used for data analysis. First-order kernel N1P1 amplitude and P1 peak time were measured. Data from individual hexagonal areas were averaged in 3 zones, according to a custom-designed pattern (Fig. 1).

Area 1 was representative of the central 5°, an area completely covered by the PDT treatment. Area 2 comprised the central 5° to 10°, an area corresponding to the border and the transition zone between the PDT-treated and the nontreated areas. The rest of the stimulated fundus (10°-25°) was designated area 3 and represented the nontreated portion of the stimulated retina. Previous reports indicate that mERG variability at the optic disc and around it is higher than the rest of the stimulated retina. Therefore, this region (consisting of 6 hexagons in area 3) was excluded from the current analysis.

All imaging tests and mERG were performed at baseline (1 week before PDT) and at 1, 3, 7, 14, 22, 28, 43, 57, 71, 84, 179, and 283 days after PDT.

**RESULTS**

**Fundus Appearance**

At day 1 after PDT, a circular region of hypopigmented retina, with a well-demarcated border corresponding to the site of photodynamic activation, was clearly visible (Fig. 2). The discoloration and whitening decreased gradually, but the border of the lesion remained visible until the end of the 9-month follow-up period. In addition, dynamic changes in the distribution, visible color, and amount of macular pigment occurred. After initial bleaching (days 1–3), gradual recovery with irreg-

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**FIGURE 1.** Schematic representation of the data averaging pattern for mERG data analysis. The zone entirely covered by the PDT treatment is indicated as Area 1, a borderline zone is labeled as Area 2, and the rest of the stimulated area is labeled as Area 3. Optic never head area (ONH) is also outlined but was excluded from the analysis because of high variability.
ularly shaped distribution was noticeable until 1 month (day 28). After that, the appearance was stable.

**Fluorescein Angiography**

Similar to the changes observed in color fundus photography, fluorescein angiography (FA) of the treated region showed a well-defined region of hypofluorescence in the early phase of FA at day 1 (Fig. 3). This was followed by hyperfluorescence in the late phase. The presence of retinal vessels over this region suggested an RPE origin of the hypofluorescent zone. However, a contribution from the underlying choriocapillaris containing damaged endothelial cells might be a contributing factor. This zone decreased in area and changed shape afterward, indicating the presence of an active RPE-remodeling process. The sequence of events was observable until day 28, and little change was detectable after that time point.

**Indocyanine Green Angiography**

ICG demonstrated an area of choroidal hypoperfusion, corresponding in size to the FA hypofluorescence area and the treated PDT region (Fig. 4). Gradual reappearance of patent choroidal vessels was observed, but the lesion remained underperfused until the end of the follow-up period. This was reflected in the ICG score for each monkey (Fig. 8).

**Optical Coherence Tomography**

Cross-section of the retina revealed some accumulation of subretinal fluid in the foveal region was present on day 1 after PDT (Fig. 5). It was even more pronounced on day 3 (data not shown) and was completely resolved by day 7 and afterward. Thinning of the retina in the foveal region (~100 μm compared with 120 μm before PDT) was present until day 283 (Figs. 5, 7).

**mfERG Results**

**N1P1 Amplitude Changes.** Both treated monkeys showed slightly different dynamics in the N1P1 amplitude mfERG changes, which correlated with changes in the amount and temporal dynamics of subretinal fluid accumulation and the degree of capillary occlusion (compare Figs. 6A, B and Figs. 7A, C). Thus, animal 9660 demonstrated more (and delayed) subretinal fluid accumulation after PDT and a higher degree of choroidal obstruction, which was paralleled by a larger spread between the N1P1 reduction of the treated area (area 1) compared with the borderline and nontreated area (Fig 7A). Nevertheless, both monkeys showed similarity in the dynamics of the N1P1 amplitude change after PDT. This parameter was reduced dramatically...
during the first week after PDT (70%–85% reduction; Fig. 7A, C). The border area (area 2) showed similar, but less pronounced (35%–40%), reduction whereas the amplitude from the nontreated area (area 3) remained at ~80% from the pretreatment values. For the period from week 1 to approximately week 9, both the treated and the border areas regained ~25% of the amplitude values. During the same period, area 3 exhibited a further decline in amplitude to ~60% of pretreatment. After an additional decline (weeks 10 and 11), area 1 stabilized to ~30% to 40% of the pretreatment values. Similar dynamics were shown in area 2, whereas area 3 showed recovery to ~50% to 70%.

P1 Peak Time Changes. Compared with the values before treatment, P1 peak time increased in up to 12 weeks (day 84) for all stimulated areas (Fig. 7). This was more pronounced for animal 121–329, whose P1 peak time in the treated area showed an 11-msec delay to an average of 35 msec (~45% delay; Fig. 7D). After that time point, the peak time values gradually returned back to pretreatment level.

Histopathologic Analysis. Various histopathologic changes were observed at 9 months after PDT. They include focal damage to the choriocapillaris characterized by areas of fibrovascular proliferation associated with closure (Fig. 8). RPE degeneration and necrosis manifesting as missing RPE nuclei and melanin redistribution in RPE cells were also observed (Fig. 8B, arrows). Localized necrosis of the photoreceptor layer manifested as thinning of the outer nuclear layer (reduced in places from 5–6 rows of nuclei to 1–2 rows of nuclei). In these areas, localized thinning of the inner nuclear layer (reduced from 8–9 rows in the control eye to 5 rows) was also observed. The inner retina and the deep layer of the choroid appeared normal. However, occasional GFAP weakly reactive cells were evident in the ganglion cell layer (data not shown).

General Health and Ocular Appearance. No changes in the conjunctiva, anterior chamber, iris, lens, or anterior vitreous were observed at any of the follow-up visits. Additionally, no skin photosensitivity was noted.

DISCUSSION
The findings of the present study confirm and extend our knowledge of the effects of verteporfin PDT on the normal
Our observation of whitening of the deep retinal layers and mild graying of the treatment area plus early hypofluorescence of the spot up to 4 weeks are in accordance with the results of previous animal and human histopathology reports. In addition, some clinical reports describe pigment epithelial changes in relatively young patients with non-ARMD CNV. Until the end of the observational period (9 months), we continued to observe hypofluorescence and some pigment mottling in the RPE on fundus photography and FA. In addition, at all time points, we recorded hypofluorescence in the corresponding region of ICG. Because ICG results from a normal retina after PDT treatment are unavailable, our observation represents a novel finding in an animal model. It correlates well with the human clinical findings reported with ICG up to 24 months after PDT and suggests permanent closure of part of the choroidal vasculature. This corresponds with the results from previous studies demonstrating vascular damage and partial vascular occlusion of the choroidal...
vessels at 1 day to 4 weeks after treatment\textsuperscript{5,31-35} and extends our knowledge about the long-term consequences of this event. Extensive closure of the normal choroid after PDT can be related (and may provide a possible explanation) to reports indicating decreased visual acuity at week 1 after PDT in some patients.\textsuperscript{54} It is generally believed that choriocapillaris hypoperfusion recovers within 3 months of PDT.\textsuperscript{35,36} However, in the present study, we observed areas of hypoperfusion even at 9 months. Although we observed irregular dysmorphic RPE cells, RPE pigment mottling, and areas of frank RPE necrosis (missing nuclei) in accordance with previous reports,\textsuperscript{17,34} multilayering of the RPE was not found during the histologic evaluation at 9 months. Various degrees of RPE damage have been observed after PDT, including RPE atrophy (after a single PDT session)\textsuperscript{35} in humans. On the other hand, though some areas in the treated eye showed considerable reduction of the outer nuclear layer (ONL) and moderate reduction in the inner nuclear layer (INL) (Fig. 8D), as a whole, the neural retina was relatively spared from damage after verteporfin PDT. Thus, the neural retina in our study appeared to be more damaged than in previous monkey verteporfin PDT experiments\textsuperscript{17} and less damaged than with hematoporphyrin-derivative PDT on monkey retina,\textsuperscript{37} in which retinal damage extended to the nerve fiber layer and was accompanied by gliosis in the neural retina. Accumulation of subretinal fluid at day 1 and day 3 after PDT has not been previously reported in experimental animals and intact retina. However, increases in foveal thickness were measured by mfERG. There was a significant initial decrease in N1P1 amplitude (up to 80% in the central part of the treated area) followed by gradual recovery up to 7 weeks after treatment and further deterioration of the response. Changes in N1P1 amplitude were paralleled by a considerable gradual increase in the implicit time of P1, possibly indicating ischemic conditions for the cellular generators of the N1P1 complex. This is similar to the delays observed in 30-Hz flicker and oscillatory potential peak times in full-field ERG in patients with diabetes.\textsuperscript{42} mfERG functional changes after PDT in nonhuman primates have not been reported. Results from mfERG testing in patients undergoing PDT for CNV are inconclusive. Some reports show no change in amplitude or timing parameters after PDT,\textsuperscript{43,44} whereas other reports indicate amplitude decreases in the area treated with PDT.\textsuperscript{45-47} Our findings are consistent with some of the later reports and show similar temporal dynamics of the mfERG amplitude deficit in the central area (considerable decrease at 1 week, partial recovery at 3 months, and persistent deficit at 6 months). Although our results were based on only 2 examined animals and they exhibited some interindividual differences, both results clearly showed the same temporal dynamics, and the magnitude of the interindividual differences were similar to those observed after other electrophysiologic tests such as human full-field ERG (Tzekov R, et al. IOVS 1998;39:ARVO Abstract 2769). A larger testing group would help clarify the range of some of the observed effects and would generate more confidence in the correlation between functional and morphologic results. Short-term changes in visual acuity\textsuperscript{40,47,48} and contrast sensitivity\textsuperscript{49} after PDT could also be correlated with deterioration of retinal neuronal function as reflected by mfERG. A key question for consideration is the apparent discrepancy between the relatively good clinical safety profile of verteporfin PDT and the various morphologic and functional toxic side effects documented in our study. Several factors may play a role. First, the accumulation of subretinal fluid accompanying CNV and the presence of CNV itself may have a protective role in sparing the normal choroidal vasculature from the occlusive effect of PDT. Subretinal fluid accumulates because of the leakiness of the CNV vessels in a substantial number of patients with ARM\textsuperscript{38,50,51} and may act as a filter absorbing some of the long-wavelength light used for photothermal activation of the drug. Second, it can also act as a singlet oxygen “trap,” allowing a sizable portion of the singlet oxygen molecules to be released in the fluid with less direct cytotoxic effects to the surrounding tissue.\textsuperscript{52} This might explain to some extent why the treatment of larger lesions was less effective through PDT.\textsuperscript{53} Theoretically, a third factor could be the use of repetitive flashes for fundus photography and FA at the day 1 and day 3 follow-up visits. They might have caused reactivation of the drug and contributed to the severity of the observed effects. To avoid delayed photosensitization, it is generally recommended that patients stay away from bright light and direct sun exposure up to 5 days after treatment. However, it has been shown that photosensitization is rare in humans (no difference has been observed in the frequency of dermatologic adverse effects from treatment with verteporfin and placebo\textsuperscript{54}), and we did not observe skin photosensitization in our
animals. Even more, the half-life of the drug in the retina is 5 to 6 hours in humans and shorter in other species. Additionally, it has been confirmed that verteporfin is cleared from the outer retina approximately 2 hours after injection. Therefore, it seems unlikely that enough drug remained at the 24-hour or the 72-hour follow-up examinations to be photoactivated and exert additional damage. A fourth factor could be the difference in melanin content. The amount of melanin in the monkey choroid is higher than in the human choroid (Oyejide A, Tzekov R, unpublished results, 2005). Melanin is usually protective from light damage, but once it is oxidized it loses this capability and may instead become cytotoxic. Our histopathologic data confirmed localized melanin pigment dispersion and clumping at 9 months, possibly explaining the substantial discoloration of the fundus after PDT. However, it is unclear to what extent the oxidation of choroidal melanin may contribute to functional and morphologic changes in the neural retina. A fifth factor could be the difference in tissue reactivity between an aged human eye, which is usually the subject of PDT treatment in a clinical setting, and a relatively younger monkey eye as used in this experiment. An indication of such difference might be the reported occurrence of RPE changes from horizontal cells or later synchronization of the response with the exception of day 84 for monkey 121-329). This might explain the far-reaching functional changes after PDT.

The magnitude and duration of PDT toxicity to the treated and untreated area are surprising. It is generally accepted that 2 main mechanisms are responsible for PDT effects: cytotoxic effects in the neighboring cells and vascular occlusion of the treated tissue (most often a neovascular formation or a tumor). Signs for both processes were observed in the present study. Activation of the RPE and Müller cells (expressed by melanin redistribution in the RPE) and glial fibrillary acidic protein (GFAP) immunoreactivity (though weak and localized to some areas) point to cytotoxic effects. Persistent closure of focal areas of the choriocapillaris, on the other hand, is direct evidence of vascular occlusion. Selective occlusion of the choriocapillaris can be explained by the faster clearance of verteporfin from the retina than from the choroid. Functional consequences of both events extended well beyond the directly treated area. This was also surprising because the retinal vasculature remained intact and the choriocapillaris was occluded only within the treated area. Although the N1P1 complex of the mfERG first-order response represents mostly interactions between hyperpolarizing and depolarizing bipolar cells, some modulation of the response by lateral interactions from horizontal cells or later synchronization of the response through the large network of ganglion cells is also possible. This is the most likely mechanism behind the observed far-reaching depression of mfERG amplitude and delayed peak time. Indeed, the observed localized thinning of the ONL may indicate damage not only to the photoreceptor but also to the horizontal cell nuclei. Additionally, GFAP activation in the inner retina and localized thinning of the INL indicate accompanying deficiencies of the inner retina. Thus, a combined suppressive effect on the horizontal and ganglion cell network might explain the far-reaching functional changes after PDT. P1 peak time values remained close to each other for all 3 areas (with the exception of day 84 for monkey 121-329). This phenomenon probably reflects stimulus-driven, long-range synchronization in the retina, similar to that recorded in the cat. The morphologic and functional PDT adverse effect observations made in this study have direct clinical relevance. Treatment parameters for the study were selected to match as closely as possible parameters used in PDT clinical trials. The eye of the cynomolgus monkey is similar morphologically and functionally to the human eye and responds similarly to PDT treatment. Although infrequent, damage to the neural retina overlaying the PDT-treated areas has been reported clinically.

The ophthalmologist performing PDT should be aware that even a single PDT session could in certain cases close the normal choriocapillaris within the treated area (outside the pathologic region) for a prolonged period. In some patients, intermittent increases in subretinal fluid level, foveal thinning, and RPE damage may be present in the same area and have long-term consequences. Finally, functional alterations of the neurosensory retina may exist even outside the treated area and have a negative effect on visual performance. In this context, the use of neuroprotective agents or anti-inflammatory agents such as corticosteroids may be beneficial in conjunction with PDT. Indeed, it was proposed recently that neurotrophic factors such as brain-derived neurotrophic factor (BDNF) may be good adjuncts to PDT and may help negate some of the adverse effects. Further work is necessary to quantify the beneficial effects of neuroprotective agents on PDT adverse effects.

References


