Selective Photodynamic Effects of the New Photosensitizer ATX-S10(Na) on Choroidal Neovascularization in Monkeys

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Objective: To determine the optimal treatment variables for photodynamic therapy (PDT) with new photosensitizer ATX-S10(Na) (13,17-bis[1-carboxypropionyl] carbamoylethyl-8-etheny-2-hydroxy-3-hydroxyminoethylidene-2,7,12,18-tetranethyl 6 porphyrin sodium) to induce selective occlusion of choroidal neovascularization (CNV) in nonhuman primate eyes.

Methods: Experimental CNV was induced in monkey eyes by laser photocoagulation, and PDT was performed in neovascularized and healthy eyes with different treatment variables. At 0 to 150 minutes after 4-, 8-, and 12-mg/kg of body weight intravenous injections of ATX-S10(Na), a diode laser was irradiated at the dose of 1 to 127 J/cm² (wavelength, 670 nm). Vascular occlusion induced by PDT was evaluated using fluorescein angiography, indocyanine green angiography, and histological examination at 1 day to 4 weeks after irradiation.

Results: Selective occlusion of CNV without damage to healthy retinal and choroidal capillaries was achieved in the following conditions: 30 to 74 J/cm² irradiation at 30 to 74 minutes after the 8-mg/kg injection, and 1 to 29 J/cm² irradiation at 30 to 74 minutes or 30 to 74 J/cm² irradiation at 75 to 150 minutes after the 12-mg/kg dye injection. Regrowth of CNV often occurred when the retina was heavily injured by excessive PDT.

Conclusion: By using optimal treatment variables, PDT using ATX-S10(Na) induces selective occlusion of CNV in nonhuman primate eyes, providing the possibility of therapeutic application to the clinical practice.

Clinical Relevance: Occlusion of CNV without direct damage to the sensory retina is useful to preserve visual acuity in patients with exudative age-related macular degeneration. A clinical trial of PDT using ATX-S10(Na) is desirable.


CHOROIDAL neovascularization (CNV) is the major cause of serious visual loss in patients with age-related macular degeneration (AMD). Although laser photocoagulation was used as the treatment modality for this disease, its thermal effect often caused considerable damage to the sensory retina, especially in the case of subfoveal lesion. Photodynamic therapy (PDT), which was introduced in cancer therapy in 1961, recently attracted much attention in ophthalmology because it is capable of occluding vessels by injuring vascular endothelial cells with a singlet oxygen emitted from laser-excited photosensitizers and because its injuring action is limited to the site of dye accumulation (unlike the thermal effect). The effectiveness of PDT on the neovascularization has been demonstrated in the cornea, iris, and choroid. Various kinds of photosensitizers have been developed. Among them, benzoporphyrin derivative (BPD) and tin ethyl etiopurpurin are superior to other agents in excretion rate and photosensitizing potency and are now used in clinical trials for CNV in AMD. However, they need to be infused in the form of liposome or emulsion because of hydrophobicity. Nakajima et al recently developed amphiphilic photosensitizer ATX-S10(Na), which can be administered as a bolus intravenous injection and thereby reduce the risk of thrombosis. Furthermore, besides the advantages common to BPD and tin ethyl etiopurpurin, ie, a desirable long absorption wavelength and rapid elimination from the body, this agent has more than 10 times the median lethal dose of polyhematoporphyrin ether/ester in mice and rats (R. Machida, PhD, unpublished data, 1999). As for biodistribution, although hydrophobic photosensi-
MATERIALS AND METHODS

ANIMALS

Twenty-four eyes from 12 cynomolgus monkeys (weight, 2.0-2.5 kg) were used. Animals were treated in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. All experimental procedures were performed while the animals were under anesthesia with intramuscular injection of ketamine hydrochloride, 50 to 60 mg/kg of body weight, and diazepam, 5 to 10 mg. Proparacaine hydrochloride was used for topical anesthesia. Pupils were dilated with 2.5% phenylephrine hydrochloride and 0.8% tropicamide.

REAGENTS

The photosensitizer ATX-S10(Na) (13,17-bis[1-carboxypropionyl] carbamoylethyl-8-etheny-2-hydroxy-3-hydroxyiminoethylidene-2,7,12,18-tetranethyl 6 porphyrin sodium) (Lederle Japan, Tokyo, and Toyo Hakka Kogyo, Okayama, Japan) was diluted with distilled water at a concentration of 10 mg/mL immediately before use. It had 2 major absorption peaks at 407 and 670 nm in the plasma.

INDUCTION OF EXPERIMENTAL CNV

Experimental CNV was induced in the posterior pole of the fundus in 22 eyes of 11 monkeys by photococagulation with a krypton laser (wavelength, 647 nm) (Novus Omni Laser; Coherent, Santa Clara, Calif) according to the method described by Ryan.27 The spot size was 75 µm in diameter, and duration of irradiation was 0.1 second. The power was 600 to 700 mW at the corneal surface. By using a fundus contact lens (IF-210R; Menicon, Nagoya, Japan), 3 to 13 burns were made in each eye, sparing the foveal center. At 10 to 24 days after photococagulation, CNV induction was confirmed by results of fundus photography and fluorescein and indocyanine green (ICG) angiography using a fundus camera and a digital videangiography system (Topcon TRC-501A plus Image Net H1024 System; Topcon, Tokyo). For fluorescein and ICG angiography, 1 mL of 5% fluorescein sodium solution and 0.5 mL of 5-mg/mL ICG, respectively, were injected intravenously.

PHOTODYNAMIC THERAPY

One day after angiography, while the animals were under anesthesia, PDT was performed in 158 CNV lesions (Table 1). The ATX-S10(Na) was injected intravenously in the doses of 4, 8, and 12 mg/kg of body weight, which had been demonstrated to be appropriate in a monkey model in our preliminary experiments. At various time intervals from 0 to 150 minutes after dye injection, eyes were irradiated for 4 minutes with a 670-nm diode laser (Hamamatsu Photonics Inc, Hamamatsu, Japan) that was connected to a 308L-M slitlamp (Carl Zeiss, Jena, Germany) with an optical fiber (diameter, 400 µm). The dim light of the slitlamp was only used at the focus irradiation and was turned off during PDT. The spot size was adjusted to cover the whole area of CNV using the aperture of a laser system (Coherent 930 Argon-Dye Laser System; Coherent). Laser irradiation was performed using a fundus contact lens (IF-210R; Menicon). The irradiance on the retinal surface was calculated from the value of a power meter (Coherent Fieldmaster; Coherent), actual spot size on the surface of retina, and transmission rate of laser irradiation in the monkey eyes, all of which had been determined in our preliminary study. The irradiance ranged from 6 to 528 mW/cm², and radiant exposures ranged from 1.4 to 126.7 J/cm². The actual spot size on the surface of retina ranged from 340 to 6800 µm in diameter.

As control experiments, 2 CNV lesions from 2 monkeys were subjected to dye injection without irradiation, and 3 lesions from 2 monkeys were irradiated by a laser of 528 mW/cm² for 4 minutes (126.7 J/cm²) without dye injection.

EVALUATION OF PDT-INDUCED CNV OCCLUSION

One day after PDT, vascular occlusion was identified using fundus photography, fluorescein angiography, and ICG angiography. Observations were repeated weekly until the end of follow-up (Table 1). For histological analysis at 1, 2, and 4 weeks after PDT, 2 eyes from 1 monkey, 6 eyes from 3 monkeys, and 12 eyes from 6 monkeys, respectively, were enucleated while the animals were under anesthesia. They were immersion fixed in Karnovsky solution at 4°C overnight, and the neovascular portion of the retina was dissected and fixed in the same fixative at 4°C overnight, postfixed in 2% osmium tetroxide for 2 hours, dehydrated in ethanol series, and embedded in epoxy resin. Semi-thin sections were stained with toluidine blue and observed by light microscopy.

EVALUATION OF PDT-INDUCED DAMAGE TO HEALTHY RETINAL AND CHOROIDAL CAPILLARIES

Using the same treatment variables as for CNV, PDT was applied to the normal regions of the fundus from 2 untreated eyes and 8 CNV-bearing eyes. The PDT-induced retinal and choroidal capillary occlusion was evaluated using results of fundus photography, fluorescein and ICG angiography, and histological analysis.
RESULTS

Photocoagulation-induced CNV was identified by ophthalmoscopy as yellowish gray subretinal lesions with serous detachment of sensory retina (data not shown), by fluorescein angiography as early- to middle-phase ring hyperfluorescence (Figure 1, A) and late-phase fluorescein leakage (Figure 1, B), and by ICG angiography as ring hyperfluorescence during the initial 5 minutes (Figure 1, C). Dye leakage in late-phase ICG angiography was negligible.

Immediately after PDT, irradiated lesions showed no ophthalmoscopic changes (data not shown). However, at day 1, they exhibited various degrees of opacity, ie, none, mild opacity in the deep retina (Figure 2, A), and dense opacity in the whole thickness of the retina (Figure 3, A), depending on the dye and irradiation doses and period after dye injection. Occlusion of CNV was represented by hypofluorescence with no ring hyperfluorescence in early- (Figure 2, B) and middle-phase (Figure 3, B) fluorescein angiography and early-phase ICG angiography (Figure 2, C), and showed little dye leakage in late-phase fluorescein angiography (data not shown). Occlusion of choriocapillaries occurred concurrently, as shown by filling defects in early-phase fluorescein angiography (Figure 2, B) and hypofluorescence in late-phase ICG angiography (data not shown). Destruction of blood-retinal barrier in the retinal pigment epithelium (RPE) was assumed from the fluorescein leakage in the boundary of irradiated areas (Figure 2, B, and Figure 3, B). Retinal arterioles and venules were also injured to various degrees, from no damage (Figure 2, B) to vascular occlusion (Figure 3, B). Histological analysis demonstrated that CNV was occluded by thrombi (data not shown). In control eyes subjected to laser irradiation or dye administration alone, there were no appreciable ophthalmoscopic and angiographic changes.

In the follow-up periods, the retinal opacity decreased, and the detachment of the sensory retina recovered ophthalmoscopically in most of the CNV-occluded lesions at later than 1 week, although pigment irregularity remained to some extent (Figure 4). Histologically, patent CNV lesions were no longer found.

Table 1. Numbers of CNV-Bearing and Healthy Eyes Used

<table>
<thead>
<tr>
<th>Dye Dosage, mg/kg</th>
<th>Total No. of Eyes (No. of Monkeys, Lesions)</th>
<th>No. of Eyes Used in Follow-up After PDT</th>
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</thead>
<tbody>
<tr>
<td>CNV</td>
<td>4 (3, 33)</td>
<td>0 0 0 4</td>
</tr>
<tr>
<td>Normal</td>
<td>3 (2, 9)</td>
<td>2 1 0 0</td>
</tr>
<tr>
<td>8</td>
<td>12 (6, 78)</td>
<td>2 2 4 4</td>
</tr>
<tr>
<td>Normal</td>
<td>4 (2, 36)</td>
<td>1 1 0 2</td>
</tr>
<tr>
<td>12</td>
<td>6 (4, 47)</td>
<td>0 0 2 4</td>
</tr>
<tr>
<td>Normal</td>
<td>3 (2, 9)</td>
<td>0 1 2 0</td>
</tr>
</tbody>
</table>

* The total number of monkeys used for the CNV experiments was 13, since 2 monkeys with CNV were used with 2 different doses in a different time. The total number of monkeys used for healthy fundus experiments was 6, since 1 monkey was used with 2 different doses in a different time. CNV indicates choroidal neovascularization; PDT, photodynamic therapy.

Figure 1. A neovascularized eye before photodynamic therapy. A, Early-phase fluorescein angiogram at 11 seconds after fluorescein dye injection. The ring hyperfluorescence indicates choroidal neovascularization in the photocoagulated portion (arrows). B, Late-phase fluorescein angiogram at 8 minutes after dye injection. Fluorescein leakage from the neovascularization is seen. C, Early-phase indocyanine green angiogram at 1 minute after dye injection. Ring hyperfluorescence (arrows) indicates neovascularization in the lesion.
In some lesions, recurrent CNV was detected by fluorescein angiography, especially at the edge of the original CNV lesions (Figure 3, C). They were larger in size than the original CNV lesions and surrounded by many macrophages and double-layered RPE cells (Figure 6). When PDT was insufficient, CNV remained patent throughout the experimental periods. Occluded retinal arterioles and venules after intensive PDT usually underwent recanalization (data not shown).

The relationship of the occlusive effect of PDT with irradiation doses and time intervals after dye injection was investigated in the 4-, 8-, and 12-mg/kg ATX-S10(Na) injections. The occlusive effect of PDT on CNV was evaluated with the assessment of patency of retinal arterioles and venules at 1 day after PDT and classified into the following 3 categories: (1) CNV closure without damage to retinal arterioles and venules (Figure 7, A-C); (2) CNV closure with damage to retinal arterioles and venules; and (3) patent CNV. Regrowth of CNV often occurred after the heavy damage to the retina by intensive PDT.

Because in this model photocoagulation injured retinal and choroidal capillaries before PDT, it was difficult to discriminate PDT-induced damage from photocoagulation-associated ones. Therefore, to examine the PDT-induced damages to the retinal and choroidal capillaries, we applied PDT to healthy retinal and choroidal tissue. Occlusion of capillaries was identified as hypofluorescence by early-phase fluorescein angiography and late-phase ICG angiography. Histological analysis demonstrated that capillaries were occluded with thrombus, and RPE cells showed swelling and disarray of photoreceptor outer segments. Retinal arterioles and venules and large choroidal vessels were not occluded (data not shown). The relationship of occlusive effect of PDT on the healthy retinal and choroidal capillaries with irradiation dose and time intervals after dye injection was investigated in 4-, 8-, and 12-mg/kg ATX-S10(Na) injections. The extent of capillary occlusion was classified into the following 3 categories: (1) choroidal and retinal capillaries closed (Figure 8, A-C); (2) choroidal capillaries alone closed; and (3) choroidal and retinal capillaries open. A follow-up study demonstrated that occluded choriocapillaries were recanalized and that the blood-brain barrier was reestablished at 1 week as shown by cessation of fluorescein leakage in the late-phase fluorescein angiography (Figure 9, A and B). In the category 2 lesions, the inner layer of the retina showed normal features, although the photoreceptor outer segments had disappeared (Figure 10). In the category 1 lesions, considerable damage remained in the inner layer of the retina.

Table 2 summarizes our results, showing the percentages of the lesions exhibiting CNV closure with no damage to sensory retina (excluding the lesions with CNV regrowth) in neovascularized eyes and those showing patency in retinal and choroidal capillaries or
in retinal capillaries alone (choroidal capillaries can be recovered from occlusion as described above) in non-neovascularized eyes. Optimal treatment conditions for providing CNV closure and little or no damage to normal retinal and choroidal capillaries in high percentages were 30 to 74 J/cm² irradiation at 30 to 74 minutes after the 8-mg/kg dye injection and 1 to 29 J/cm² at 30 to 74 minutes or 30 to 74 J/cm² at 75 to 150 minutes after the 12-mg/kg dye injection (although the amount of normal tissue used for PDT with 1 to 29 J/cm² in the 12-mg/kg dye injection was small, retinal capillaries that were always patent, independent of time intervals). In the 4-mg/kg dye injection, a high percentage of CNV closure and little or no damage to normal retinal and choroidal capillaries in high percentages were 30 to 74 J/cm² irradiation at 30 to 74 minutes after the 8-mg/kg dye injection and 1 to 29 J/cm² at 30 to 74 minutes or 30 to 74 J/cm² at 75 to 150 minutes after the 12-mg/kg dye injection (although the amount of normal tissue used for PDT with 1 to 29 J/cm² in the 12-mg/kg dye injection was small, retinal capillaries that were always patent, independent of time intervals).
closure was obtained by 30 to 74 J/cm² irradiation at 30 to 74 minutes, but, as shown in Figure 7, A, the range of effective CNV closure was much narrower compared with the above-mentioned optimal conditions.

**COMMENT**

This study has shown that PDT with ATX-S10(Na) successfully induces a long-term (up to 4 weeks), selective CNV occlusion in primate eyes, sparing damage to sensory retina. This closure is considered to result from a photodynamic effect but not from a thermal effect, because the irradiance used here, 528 mW/cm² at maximum, does not induce thermal coagulation; laser irradiation alone did not induce the closure; and no CNV damage occurred immediately after irradiation as mentioned by others.¹⁶

Optimal treatment conditions for selective PDT were determined by CNV closure (excluding the case of CNV regrowth) in neovascularized eyes and patency of retinal and choroidal capillaries or retinal capillaries alone in nonneovascularized eyes, because occluded choroidal capillaries, unlike retinal ones, can be recanalized later. As indicated in Table 2, selective occlusion of CNV was achieved by 30 to 74 J/cm² irradiation at 30 to 74 minutes after the 8-mg/kg dye injection and by 1 to 29 J/cm² at 30 to 74 minutes or 30 to 74 J/cm² at 75 to 150 minutes after the 12-mg/kg dye injection. In the 4-mg/kg ATX-S10(Na) injection, the range of conditions for selective occlusion was much narrower in laser irradiance and time elapsing after dye injection compared with the 8- and 12-mg/kg dye injections, whereas the dye dose of 12 mg/kg produced the widest range. Although Kramer et al¹⁷ suggested, based on their experiment using liposomal BPD, that reduction of dye dose could increase the selectivity of PDT, our results indicate that the smaller the dye dose, the narrower the range of optimal treatment conditions becomes.

A previous study using a rat model¹⁵ demonstrated that CNV was selectively occluded by laser irradiation performed at 2 to 4 hours after ATX-S10(Na) injection. Selectivity at this period was considered to result from heavy accumulation of ATX-S10(Na) in the neovascular tissue and diminishment of dye in healthy tissue, including the sensory retina as shown by fluorescence microscopy. Although kinetics and localization of ATX-S10(Na) accumulation in monkey CNV have not been fully elucidated, our recent work on ATX-S10(Na) fundus angiography supports the idea that dye is preferentially accumulated in the neovascular tissue at the optimal periods for PDT.

Healthy choriocapillaries were often occluded by PDT in optimal conditions for selective occlusion of CNV, and RPE cells were also damaged. Similar damage
to choriocapillaries and RPE cells was observed in PDT using BDT, even in optimal conditions for selective occlusion of CNV,\textsuperscript{15-17} so our results seem to be comparable to those of BPD-PDT. Choriocapillaries recovered from injuries at 1 week, although retinal damage, such as double-layered RPE cells and loss of photoreceptor outer segment, persisted. Although retinal damage was much milder than that caused by laser photocoagulation, the functional alterations associated with these events are not known and need to be investigated.

Regrowth of CNV is the major problem in the clinical trial of PDT using BPD for AMD.\textsuperscript{20,21} It was distinguished from recanalization of original CNV by larger size and a different ring-shaped pattern of vessels in early-phase fluorescein angiography. Although the mechanism of regrowth is not known, it is assumed that feeder vessels of CNV surviving after PDT may be responsible.\textsuperscript{28} Our observation that large choroidal vessels usually remained open is consistent with this view. The possibility of regrowth from residual neovascularization after insufficient PDT is excluded, because regrowth more frequently occurred after excessive irradiation than in optimal treatment conditions. Furthermore, regrowth was usually observed when retinal arterioles and venules were damaged. From these observations, the following 3 causes for regrowth are postulated: (1) inflammation evoked by excessive PDT, (2) ischemia of the sensory retina after vascular occlusion, and (3) destruction of RPE cells covering CNV. The PDT-induced damage of retinal vessels, RPE cells, and choriocapillaries induces infiltration of macrophages, which may produce vascular endothelial growth factor\textsuperscript{29} and contribute to induction of CNV.\textsuperscript{30} It has been reported that RPE cells promote endothelial prolifera-

Figure 8. The relationship of injuring effects of photodynamic therapy (PDT) on normal retinal and choroidal capillaries with irradiation dose and time interval after dye injection in doses of 4 (A), 8 (B), and 12 (C) mg/kg at 1 day after PDT. The degrees of vascular occlusion by PDT were classified into the following 3 categories: choriocapillaries closed but retinal capillaries open (solid circles); choriocapillaries and retinal capillaries closed (Xs); and choriocapillaries and retinal capillaries open (open circles).

Figure 9. Nonneovascularized eyes at 1 week after photodynamic therapy with 8-mg/kg injection of ATX-S10(Na). Lesion 1 was irradiated by a dose of 40 J/cm\textsuperscript{2} at 45 minutes after dye injection; lesion 2, 30 J/cm\textsuperscript{2} at 50 minutes; lesion 3, 50 J/cm\textsuperscript{2} at 55 minutes. A, Middle-phase fluorescein angiogram at 23 seconds after dye injection. A hypofluorescent lesion at the center of the treated area, representing the occlusion of choriocapillaries, is small. Retinal vessels are well perfused. B, Early-phase indocyanine green angiogram at 2 minutes 28 seconds after dye injection. Choroidal large vessels are well perfused.
Photodynamic therapy using ATX-S10(Na) with a diode laser effectively and selectively occludes CNV in non-human primate eyes under optimal treatment conditions and may be a promising modality for the treatment of patients with AMD. Focal irradiation is important for preventing the regrowth of CNV.

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REFERENCES


Table 2. Lesions With Effective CNV Closure in Neovascularized Eyes and Those With Normal Retinal Capillaries Not Occluded in Nonneovascularized Eyes at Various Intervals After PDT*

<table>
<thead>
<tr>
<th>Dose, mg/kg</th>
<th>Fluence, J/cm²</th>
<th>Time After Irradiation, min</th>
<th>Effective Occlusion of CNV†</th>
<th>No Occlusion of Normal Retinal Capillaries‡</th>
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<tr>
<td></td>
<td></td>
<td>0-29</td>
<td>30-74</td>
<td>75-150</td>
</tr>
<tr>
<td>4</td>
<td>1-29</td>
<td>2/8  (25)</td>
<td>0/1  (0)</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>30-74</td>
<td>1/4  (25)</td>
<td>3/4  (75)</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>75-150</td>
<td>1/6  (17)</td>
<td>2/6  (33)</td>
<td>NT</td>
</tr>
<tr>
<td>8</td>
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<td>2/17 (12)</td>
<td>1/20 (5)</td>
<td>0/5  (0)</td>
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<tr>
<td></td>
<td>75-150</td>
<td>0/1  (0)</td>
<td>4/13 (31)</td>
<td>2/6  (33)</td>
</tr>
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</table>

* CNV indicates choroidal neovascularization; PDT, photodynamic therapy; and NT, not tested. Bold face type highlights values = 75%. Data are given as number/number of examined lesions (percentage).
† Indicates CNV closure without damage to retinal arterioles and venules and does not include CNV regrowth.
‡ Indicates patency of retinal and choroidal capillaries or retinal capillaries alone.

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