PDT to Monkey CNV with ATX-S10(Na): Inappropriateness of Early Laser Irradiation for Selective Occlusion

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PURPOSE. There is controversy about which mode of laser irradiation, early irradiation with low-dose photosensitizer or late irradiation with high-dose, benefits the selective occlusion of choroidal neovascularization (CNV) in photodynamic therapy (PDT). In this study, using an amphiphilic photosensitizer, 13,17-bis (1-carboxypropionyl) carbamoylhexyl-8-ethene-2-hydroxy-3-hydroxyminoethylidene-2,7,12,18-tetraethyl porphyrin sodium (ATX-S10(Na); Photochemical Inc., Okayama, Japan), photodynamic and adverse effects of early irradiation on CNV-bearing monkey eyes were investigated.

METHODS. Experimentally induced CNV lesions and normal retina were irradiated with a diode laser (670-nm wavelength) at a dose of 1 to 90 J/cm² at 1 to 19 minutes after intravenous injection of 2 mg/kg body weight of ATX-S10(Na). Vascular occlusion and CNV recurrence were evaluated by fluorescein and indocyanine green angiography and histologic analysis, until 4 weeks after irradiation.

RESULTS. Of 45 different conditions, 23 did not induce CNV closure, 20 provided both CNV occlusion and retinal vessel damage, and 2 achieved selective CNV occlusion without retinal vascular injury. Recurrence of CNV was induced in 19 of 22 CNV-occluding conditions. ATX-S10(Na) angiography showed that dyes were similarly distributed between normal vessels and CNV at early time periods after injection, whereas they were preferentially accumulated in CNV after 30 minutes.

CONCLUSIONS. In PDT with ATX-S10(Na), irradiation within 20 minutes of dye injection failed to induce selective CNV occlusion, probably because there is no significant difference in the biodistribution of dye between CNV and retinal vessels. It also caused frequent CNV recurrence after extensive inflammation in the irradiated retina. (Invest Ophthalmol Vis Sci. 2001;42:2639–2645)

In patients with age-related macular degeneration (AMD), choroidal neovascularization (CNV) often causes severe visual loss. Laser photocoagulation has long been used as the therapy for this pathologic event,1 but it has shown limited efficacy, depending on the site of CNV occurrence in the fundus. For example, when CNV appears in the subfoveal region, this therapy often causes a sudden visual loss due to thermal coagulation-related injuries in the sensory retina.2 Other treatment modalities, such as radiation therapy2 and surgical removal of CNV,3 do not always give satisfactory results, and, furthermore, the effectiveness of macular translocation surgery3 still remains unclear.

At present, photodynamic therapy (PDT) is the most promising modality for CNV occlusion. Five photosensitizers are currently under investigation.6–17 They are categorized into three groups of dyes: lipophilic, hydrophilic, and amphiphilic. Lipophilic dyes include benzoporphyrin monoacid (BPD-MA)8–15 and tin-ethyl etiopurpurin (SnET2),16 the former of which is the only agent approved for clinical use in the treatment of CNV in AMD,13–15 and the latter of which is undergoing testing in a phase III clinical trial. Compared with placebo, BPD-MA shows a significantly higher rate of visual preservation in patients with predominantly classic CNV, in which classic CNV occupies more than 50% of the neovascular lesion13–15.

In the clinic, for intravenous administration, lipophilic photosensitizers are used in the form of liposomes. Hydrophilic photosensitizers include lutetium texaphyrin and mono-L-aspartyl chlorin e6,17 the former of which is undergoing testing in a phase-I/II clinical trial and the latter of which is in the preclinical stage. Amphiphilic dyes that possess both lipophilic and hydrophilic properties include a novel photosensitizer ATX-S10(Na), which we have recently developed as a potent agent for PDT.18–21

Differences in chemical structures among photosensitizers lead to differences in subcellular localization in the target cell. Liposomal BPD-MA and SnET2 are bound to low-density lipoproteins (LDLs) in the blood stream, taken up by the target cell through LDL receptor-mediated endocytosis, and diffusely distributed in the cytoplasm.22 Hydrophilic dyes are taken up by the target cell through endocytosis and preferentially accumulate in the lysosomes. An amphiphilic dye ATX-S10(Na) is conjugated with high-density lipoproteins (HDLs), albumin, and other plasma proteins in the blood; is incorporated by the target cell through endocytosis; and accumulates mainly in the lysosomes23 and, in part, in the cell membrane and membranous organelles (Obana et al., unpublished data, 2001). There are also differences in the kinetics and tissue distribution after in vivo administration. Whereas liposomal BPD-MA is rapidly taken up by vascular endothelial cells and soon disappears from them, leaving a large deposit of dyes in the retinal pigment epithelium (RPE),24,25 ATX-S10(Na) more gradually accumulates in the neovascular wall with a peak at 1 hour, leaving little accumulation in RPE.19

In our recent study, for a clinical trial of PDT with ATX-S10(Na) in humans, we determined the optimal timing and dose of irradiation for the selective occlusion of experimental CNV in monkey eyes.21 The results demonstrated that laser irradiation at 30 to 74 minutes after injection of 4 or 8 mg/kg BW of ATX-S10(Na) and at 30 to 150 minutes after injection of 12 mg/kg BW of dye effectively closes CNV, with minimal
damage to the healthy retinal and choroidal vessels surrounding the lesion. Several previous studies using other photosensitizers indicated that the optimal timing for laser irradiation is early: 5 to 30 minutes after dye injection for chloroaluminum phthalocyanine,7 within 5 minutes for mono- asparyl chlorin e6,17 and 15 minutes for BPD-MA.15 In the preclinical studies of BPD-MA, irradiation between 20 and 50 minutes has been suggested to be appropriate.10 The advantages of such early irradiation might come from the lower doses of dye and irradiation required. It is also reported that laser irradiation at later than 30 minutes causes dye-leakage-related injuries in the sensory retina.8,25 In this study, to determine whether PDT with ATX-S10(Na) has a similar benefit in early laser irradiation, we compared the photodynamic effect on CNV occlusion and the adverse effects such as injuries to retinal vessels and recurrence of CNV between the PDT with early irradiation (1–19 minutes after dye injection) in a low dose (2 mg/kg BW) of ATX-S10(Na) and PDT with late irradiation (later than 30 minutes) in high doses (4, 8, and 12 mg/kg BW).21

MATERIALS AND METHODS

Animals
Nine cynomolgus monkeys (2–2.5 kg) were used. They were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental procedures were performed with monkeys under anesthesia with intramuscular injection of 50 to 60 mg/kg BW ketamine hydrochloride and 5 to 10 mg of diazepam. Preparacaine HCl was used for topical anesthesia. Pupils were dilated with 2.5% phenylephrine hydrochloride and 0.8% tropicamide.

Induction of Experimental CNV
Experimental CNV was induced in the posterior pole of the fundus by photocoagulation with krypton laser (wavelength, 647 nm; Novus Omni Laser; Coherent, Santa Clara, CA). At 14 to 31 days after photocoagulation, CNV was confirmed by ophthalmoscopy, sodium fluorescein (SF) angiography, and indocyanine green (ICG) angiography, as previously described.21

Photosensitizer
The photosensitizer 13,17-bis (1-carboxypropionyl) carbamoyethylethynyl-2hydroxy-5-hydroxyiminooctahydridene-2,7,12,18-tetraethyl porphyrin sodium (ATX-S10(Na), Photochemical Inc., Okayama, Japan) is an iminochlorine aspartic acid derivative. The dye was diluted with distilled water into the concentration of 10 mg/ml before use. This dye has an absorption peak at a wavelength of 401 nm in the soret band and at 664 nm in the Q band in a water solution. Both absorption peaks shift to the longer wavelength in the plasma solution, and the peak in the Q band in the plasma measures 670 nm. When the dye in the plasma solution is excited with a 670-nm light, it emits fluorescence at a maximum wavelength of 680 nm.

ATX-S10(Na) Angiography
Six monkeys were divided into two groups. Each three animals received an intravenous injection of 4 mg/kg and 8 mg/kg BW ATX-S10(Na) as had been used in the previous study.21 (Although the dye dosage of 2 mg/kg was also tried, clear fluorescence images were not obtained [data not shown].) The fluorescence of ATX-S10(Na) from the fundus was detected by a modified fundus camera (TRC-50IA; Topcon, Tokyo, Japan) which included a 670-nm wavelength diode laser (Hamamatsu Photonics Inc., Hamamatsu, Japan) as the stimulation source and a cooled charge-coupled device (CCD) camera (KP-160; Hitachi, Ibaragi, Japan) as a detector. A sharp-cut filter (SC70; Fuji, Kanagawa, Japan) blocked the light of wavelengths shorter than 670 nm and was used as a barrier filter. Laser irradiance was 9.1 to 15.5 mW/cm², as measured on the corneal surface with a power meter (Nova-Display; Ophir Optronics, Inc., Boston, MA). The exposure time for taking angiographic photographs was less than 10 seconds, which was too short to cause photodynamic injury to the retinal tissue. Autofluorescence was absent in the fundus before dye injection. The data were recorded on S-VHS video tape and/or a digital video system. Angiograms were performed on both eyes at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 30, 40, 50, and 60 minutes and 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, and 24 hours after dye injection.

PDT on CNV
PDT was performed on 45 lesions of CNV in six eyes in three monkeys under general anesthesia. At 1 to 19 minutes after intravenous injection of 2 mg/kg BW ATX-S10(Na), laser irradiation of 6.9 to 437.5 mW/cm², as calculated on the retinal surface, which does not induce thermal coagulation, was conducted for 30 to 240 seconds (radiant exposure was calculated to be 1–86.9 J/cm²), using a slit lamp system equipped with a 670-nm diode laser (Hamamatsu Photonics Inc.) and a fundus contact lens (IF-210R; Menicon, Nagoya, Japan). The spot size on the retinal surface was 1500 to 3000 μm in diameter, which covered the whole area of CNV. Laser power was checked before every experiment by a power meter (Fieldmaster; Coherent). As controls, two lesions of CNV from two monkeys were subjected to dye injection without irradiation, and three lesions from two monkeys were irradiated by a laser at 437.5 mW/cm² (61.3 J/cm²) for 140 seconds before dye injection.

PDT on the Healthy Retina and Choroid
PDT was performed on five areas of the normal chorioretina in two eyes of one monkey. At 1, 3, 5, 9, and 11 minutes after intravenous injection of 2 mg/kg BW ATX-S10(Na), laser irradiation of 437.5 mW/cm² was conducted for 60 to 120 seconds with a radiant exposure of 26.3, 26.3, 32.8, 39.4, 52.5 J/cm², respectively, with a spot diameter of 2000 μm.

Evaluation of Vascular Occlusion
One day after PDT, vascular occlusion was identified by SF and ICG angiography. Observations were made every week for healthy and neovascularized regions until 3 and 4 weeks after testing, respectively. Monkeys were then killed by intravenous injection of an overdose of pentobarbital sodium, and the eyes were enucleated and fixed in Karnovsky fixative overnight at 4°C. Semi-thin sections were stained with toluidine blue and observed by light microscopy.

RESULTS

Time Course Analysis on the Distribution of ATX-S10(Na)
In ATX-S10(Na) angiography, bright fluorescence appeared in the choroid at 6 seconds after dye injection. Dye then appeared in the retinal arteries on the optic disc and in the venous return within the next few seconds. The choroidal vessels were more clearly seen by ATX-S10(Na) angiography (Fig. 1A) than by SF angiography (Fig. 1C). Dye infusion in the short and long posterior ciliary arteries, however, was less obviously demonstrated by ATX-S10(Na) angiography (Fig. 1A) than by ICG angiography (Fig. 1D). Hyperfluorescence in ATX-S10(Na) angiography representative of CNV lesions was observed within 10 seconds and gradually increased in intensity (Fig. 1A), although the dye leakage shown by ATX-S10(Na) angiography was less obvious with SF angiography (Fig. 1C). At 10 to 20 minutes, ATX-S10(Na) fluorescence in the retinal capillaries had faded away, whereas that in the retinal arteries and veins persisted. Hyperfluorescence in the CNV was still apparent,
showing a mild degree of dye leakage (data not shown). At later than 30 minutes, whereas dye fluorescence in the retinal arteries and veins was diminished, that in the CNV persisted until 90 minutes and 120 minutes, with dye doses of 4 and 8 mg/kg, respectively (Fig. 1B), and then declined at 180 minutes. By 24 hours, ATX-S10(Na) fluorescence entirely diminished in all the structures of chorioretinal tissues.

Effects of PDT. By ophthalmoscopy, the CNV present before PDT was seen as yellowish gray subretinal proliferative tissue (data not shown) with ring-shaped hyperfluorescence showing late dye leakage in SF angiography (Fig. 2A) and ring hyperfluorescence with negligible amounts of late dye leakage in ICG angiography (data not shown). Immediately after PDT, no obvious changes were found in ophthalmoscopy. At 1 day after irradiation, 22 of 45 irradiated CNV lesions were occluded as indicated by whitish opacity with a grayish white halo in the full thickness of retina shown by ophthalmoscopy (Fig. 2B) and disappearance of ring hyperfluorescence with late dye leakage shown by SF angiography (Fig. 2C) and ICG angiography (Fig. 2D). Choriocapillary occlusion, as indicated by hypofluorescence in SF angiography (Fig. 2C) and ICG angiography (Fig. 2D), was concurrently detected. Moreover, damage to the blood-retinal barrier in the retinal pigment epithelial (RPE) cells occurred, as represented by fluorescein leakage at the margin of lesions in SF angiography (Fig. 2C). In contrast to choriocapillaries and CNV, large choroidal vessels were well perfused in ICG angiography (Fig. 2D).

In 20 of 22 CNV-occluded lesions, the retinal arterioles and venules were not occluded but exhibited fluorescein leakage from the vessels, whereas the retinal capillaries were closed (Fig. 2C). In the remaining two lesions, no damage to retinal vessels was seen. Among 23 CNV lesions without CNV closure, some lesions with mild opacity in ophthalmoscopy displayed fluorescein leakage as the result of a break in the blood-retinal barrier in the RPE, whereas other lesions with no opacity showed no apparent changes in SF and ICG angiography. At 1 week after irradiation, retinal opacity decreased in intensity in
the lesions, and dye leakage disappeared from the retinal arterioles and venules in SF angiography. However, at 1 to 4 weeks, ring-shaped hyperfluorescence with late dye leakage, which represented recurring neovascularization, appeared in some lesions in SF (Fig. 3A) and ICG (Fig. 3B) angiography. Histologic analysis demonstrated that new vessels were formed in the subretinal proliferative tissue and that underlying choriocapillaries were not occluded (Fig. 4A, arrow in inset). In the irradiated region adjacent to the subretinal proliferative tissue, pigment-laden cells overlay the RPE (Fig. 4B).

In control eyes subjected to laser irradiation alone or dye administration alone, there were no appreciable ophthalmoscopic or angiographic changes.

Figure 5 summarizes the efficacy of the PDT that was conducted at 1 to 20 minutes after administration of 2 mg/kg BW of ATX-S10(Na). Among 22 CNV-occluded lesions, 20 lesions displayed damage to retinal arterioles and venules. It was therefore difficult to clearly delineate the zone of optimal treatment conditions for selective CNV occlusion. Moreover, CNV re-

**Figure 3.** The same eye as shown in Figure 2, at 2 weeks after PDT. (A) SF angiography showed ring-shaped hyperfluorescence, with dye leakage indicative of CNV regrowth in both lesions (arrow and arrowhead). (B) ICG angiography showed the neovascular nets of hypofluorescence (arrow and arrowhead) inside the lesions.

**Figure 4.** Light micrograph of the CNV lesion (A, arrowhead) and the region adjacent to the CNV at 4 weeks after PDT. (A) In the proliferative subretinal tissue were found patent new vessels (arrow in inset). (B) Pigment-laden cells overlying the RPE. The choriocapillaries were open. Toluidine blue-staining. Original magnification, (A, B) ×50; (A, inset) ×250.
curred in 19 lesions, including the two with selective CNV occlusion.

Influence of PDT on the Normal Retina and Choroid

To more clearly demonstrate the adverse effect of PDT on the normal tissue surrounding neovascular lesions, we examined PDT-induced changes in normal eyes. Immediately after PDT, irradiated lesions showed no changes on ophthalmoscopy. At day 1, a color change from grayish to whitish was observed in ophthalmoscopy. Treatment conditions that induced CNV closure in the above experiments caused hypofluorescence indicative of choriocapillary occlusion in the normal eyes in early-phase SF and ICG angiography in four of five lesions (Fig. 6). Fluorescein leakage from the retinal arterioles and venules was also noted. Hyperfluorescence was induced in all the irradiated lesions in late-phase SF angiography (data not shown). One lesion that was treated with a laser exposure of 26.3 J/cm² 3 minutes after dye injection showed the occlusion of large choroidal vessels in ICG angiography (data not shown).

At 1 week, although fluorescein leakage from the retinal arterioles and venules was no longer seen, choriocapillary occlusion persisted. At 2 to 3 weeks, PDT-treated lesions showed pigment mottling in ophthalmoscopy. Choriocapillary occlusion, as represented by hypofluorescence in early-phase SF angiography, was no longer seen, and mottled hyperfluorescence and hypofluorescence were noted instead. In late-phase ICG angiography, hypofluorescence was observed in treated areas. Histology demonstrated that RPE preserved its original structure of a single cell layer (Fig. 7, inset). Many macrophages were accumulated on the apical side of the RPE, representing extensive inflammation. They vigorously incorporated pigments, which may have interfered with the fluorescence of ICG. The choroid showed normal architecture with patent choriocapillaries. Retinal vessels were also open. Outer segments of photoreceptors became shortened or were absent in part. Most of the nuclei in the outer nuclear layer were weakly stained with toluidine blue, and some showed pyknotic changes. Nerve fibers and ganglion cell layers were normal in appearance.

DISCUSSION

In PDT for cancer, to obtain a selective effect, laser irradiation is conducted at the time point when photosensitizers are more preferentially accumulated in the cancerous tissue than in the surrounding normal tissue. For instance, in PDT with hematoporphyrin derivatives, the optimal time point for irradiation is 48 to 72 hours after dye injection when tumor cells and endothelial cells of tumor capillaries vigorously incorporate...
dyes. In the ophthalmology, however, there remains controversy about whether laser irradiation immediately or at some interval after dye injection exerts a selective injuring effect on CNV. The proposal of immediate irradiation comes from the belief that high concentrations of blood-borne dye achieved at an early time has the maximal photodynamic effect on vascular endothelial cells by generating a large amount of free radicals, even with low doses of photosensitizer and irradiation. In fact, in PDT with chloroaluminum sulfonated phthalocyanine and mono-L-aspartyl chlorin e6, laser irradiation at 5 to 30 minutes and 5 minutes, after dye injection, respectively, yields the maximal occluding effect on CNV. In PDT with BPD-MA as well, irradiation is conducted at 5 minutes after the end of 10-minute dye infusion. Moreover, late irradiation may exert an adverse effect on the RPE and sensory retina by causing dye leakage from CNV and the choriocapillaris.

We previously observed in PDT with ATX-S10(Na) that laser irradiation at 30 to 74 minutes after injection of 4 or 8 mg/kg BW of dye and at 30 to 150 minutes after 12 mg/kg BW of dye induced a highly selective CNV closure with minimal damage to surrounding healthy retinal and choroidal vessels. As demonstrated in this study, early irradiation (1–19 minutes) with a low dose (2 mg/kg BW) of ATX-S10(Na), however, failed to achieve selective CNV occlusion without neovascular recurrence; 43 of 45 conditions did not induce selective CNV closure (although concurrent damage to retinal vessels and normal chorioretina was repaired within 2–3 weeks) and the remaining two conditions induced selective CNV occlusion but caused CNV recurrence.

The selectivity of PDT was closely related to the biodistribution and kinetics of dye accumulation in the tissue, which seem to depend on the chemical properties of dye. In this study, ATX-S10(Na) angiography demonstrated that, at the dose of 8 mg/kg BW, dye accumulation in CNV persisted until 120 minutes, whereas that in the retinal capillaries and retinal arteries and veins persisted until 30 minutes, indicating that the dyes accumulated more in CNV between 30 and 120 minutes. This time interval was compatible with that of laser irradiation for selective CNV occlusion. At a dose of 2 mg/kg BW, although no clear fluorescence images were obtained by angiography (data not shown), distribution and kinetics of dye accumulation was considered to be comparable. Nonselectivity of the injuring effect of early PDT with 2 mg/kg BW ATX-S10(Na) was considered to result from the absence of preferential accumulation of dye in CNV lesions at this time point. It was also noted that the dye leakage from the CNV was less appreciable in ATX-S10(Na) angiography than in SF angiography, which may be related to the higher affinity of ATX-S10(Na) to the plasma proteins. The property of little leakage from the CNV will decrease the adverse effect of PDT on the sensory retina.

The recurrence of CNV is a major clinical problem in PDT. This pathologic event is considered to involve not only recanalization but also regrowth, because the recurring CNV lesion is usually larger than the original lesion, as shown in Figures 2A and 3A. In the present study, CNV recurrence occurred in 19 of 22 CNV-occluding conditions and was usually seen in the lesions where retinal injuries (i.e., closure of capillaries, damage to the retinal arterioles and venules, and a break of the blood-retinal barrier) were extensive. Tissue in-
juries and the hypoxia in the sensory retina induce angiogenesis through macrophage accumulation and vascular endothelial growth factor (VEGF) production. Proliferation of VEGF-expressing fibroblasts has been demonstrated in AMD. In this study, early PDT provoked intense inflammation in the retina, which may have led to a recurrence of the CNV, probably mediated by VEGF.

Between the human AMD and monkey CNV examined in this study, there must be differences in pathogenesis; the former disorder is degenerative, whereas the latter includes potent inflammatory responses. The optimal condition for selective PDT in human AMD may accordingly differ from that obtained in our present and previous studies and therefore should be determined in a future clinical trial. The present data, however, strongly suggest that, when applied in human AMD, early laser irradiation (within 20 minutes after injection), even with low-dose ATX-S10(Na), similarly fails to achieve selective CNV occlusion and produces a higher incidence of CNV regrowth. Laser irradiation at later than 20 minutes is thus recommended.

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References