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Accumulation of Photosensitizer ATX-S10 (Na) in Experimental Corneal Neovascularization

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Purpose: To determine the most appropriate time for laser irradiation to produce selective occlusion of new corneal vessels by photodynamic therapy (PDT) with a new photosensitizer, ATX-S10(Na).

Methods: The time course of the plasma levels of ATX-S10(Na) and the degree of dye accumulation in the corneal neovascularization after intravenous administration was determined in rabbit eyes. Plasma concentration of ATX-S10(Na) was analyzed by a spectrophotometer. The amount of ATX-S10(Na) in the new corneal vessels was measured by nitrogen-pulsed laser spectrofluorometry. Frozen sections of neovascularized cornea and iris were observed by fluorescence microscopy.

Results: Plasma ATX-S10(Na) concentration was highest 5 minutes after dye injection and rapidly decreased and reached almost zero at 24 hours, indicating its prompt excretion from the body. The amount of ATX-S10(Na) in the new corneal vessels as measured by nitrogen-pulsed laser spectrofluorometry increased and reached maximal level at 2 to 4 hours. Under fluorescence microscopy, the dye was more abundantly localized in the wall of new corneal vessels than in the normal tissue at 2 to 4 hours.

Conclusion: These results indicate that laser irradiation between 2 and 4 hours after dye injection is appropriate for selective PDT with ATX-S10(Na) for the occlusion of new corneal vessels. **Jpn J Ophthalmol 2000;44:348–353** © 2000 Japanese Ophthalmological Society.

Key Words: ATX-S10(Na), corneal neovascularization, dye accumulation, fluorescence microscopy, photodynamic therapy.

Introduction

Photodynamic therapy (PDT) consists of the intravenous administration of a photosensitizer and the subsequent laser irradiation at the absorption peak of the photosensitizer. Photodynamic therapy has been used for the treatment of tumors[1] because it has been shown that the anti-tumor effect of PDT is due to ischemia resulting from destruction of tumor vasculature.[2] In ophthalmology, interest has developed in PDT as a new method of treatment for ocular neovascularization.[3–9]

Corneal neovascularization is associated with various corneal diseases, such as infectious corneal ulcers and chemical burns, and can lead to a decrease of visual acuity due to corneal opacity. Corneal neovascularization is also a problem after corneal transplantation because of its promotion of rejection reaction to the corneal graft. Although laser photocoagulation has been used to occlude new corneal vessels, there have been some problems, such as corneal thinning, hemorrhage into the cornea,[10,11] and iris damage. In addition, there is no lasting improvement in visual acuity, especially in the patients with extensive neovascularization.[12]

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We previously reported that PDT using hematoporphyrin derivative was effective for the occlusion of vessels in experimental corneal neovascularization.[8] However, as the retention time of hematoporphyrin is long, patients had to remain in a relatively dark environment for a few weeks after injection to avoid skin photosensitization. Recently, second-generation photosensitizers have been developed. They are eliminated more quickly from the body and cause fewer side effects than the first-generation photosensitizers. Among these agents, benzoporphyrin derivative[13] and tin ethyl etiopurpurin are currently undergoing clinical trials as PDT sensitizers to treat choroidal neovascularization. A second-generation photosensitizer, ATX-S10(Na), developed by Nakajima et al,[14,15] is water-soluble and is thus different from benzoporphyrin and tin ethyl etiopurpurin which are lipid soluble and bound to liposomes. The water-soluble ATX-S10(Na) can be administered intravenously as an aqueous solution.

We previously conducted experiments on the treatment of choroidal neovasularization by photodynamic therapy using ATX-S10(Na), and found that the optimal treatment conditions for the selective occlusion of new corneal vessels were dependent on three parameters: the dye dosage, the laser irradiation dosage, and the time of laser irradiation after dye injection.[16,17] For example, with reference to the time of irradiation, a time interval of a few hours might be required to allow the photosensitizer to disappear from the normal tissue while remaining in the neovascularized tissue.

In the present study, the accumulation of ATX-S10(Na) in the neovascularized and surrounding normal corneal tissue was analyzed by using nitrogen-pulsed laser spectrofluorometry and fluorescence microscopy in a rabbit model. The plasma concentration of ATX-S10(Na) was also measured. The optimal timing for starting the photoactivation for selective occlusion of choroidal neovascularization was determined.

Materials and Methods

Photosensitizer

ATX-S10(Na) (13,17-bis [1-carboxypropionyl] carbamoylethyl-8-ethenyl-2-hydroxy-3-hydroxyimino-ethylidene-2, 7,12, 18-tetramethyl porphyrin sodium; molecular weight = 927.79) (Figure 1) was supplied by the Photochemical Company (Okayama). This dye has the highest absorption peak at wavelengths of 407 nm and a second peak at 670 nm. It emits fluorescence at 680 nm wavelength when excited by 407 nm light. The ATX-S10(Na) powder was dissolved in distilled water at a concentration of 10 mg/mL immediately before use.

Experimental Corneal Neovascularization

Albino rabbits (2.5–3.0 kg body weight) were anesthetized with an intramuscular injection of ketamine hydrochloride (15 mg/kg body weight) and xylazine hydrochloride (1.5 mg/kg body weight). Proparacaine hydrochloride was used for topical anesthesia. Neovascularization was induced in both eyes by the intracorneal suture technique. Three interrupted sutures were placed with 7/0 silk, starting from the 10, 12, and 2 o'clock positions of the corneal limbus toward the center, without burying the knots.[7] Dibekacin sulfate solution was applied at the end of the procedure.

After 7–10 days, when new vessels reached the end of the sutures, the following experiments were begun. All procedures were done in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Plasma Level of ATX-S10(Na)

Three rabbits were injected intravenously with 16 mg/kg body weight of ATX-S10(Na) into a marginal ear vein. Blood samples were taken from a vein on the opposite side of the ear at 5, and 30 minutes, and 1, 2, 3, 4, 5, 6, 12, and 24 hours after dye administration. The plasma was separated by centrifugation and analyzed by a spectrophotometer (UV-1200; Shimadzu, Kyoto). The concentrations were obtained by comparison with a standardized curve.

ATX-S10 (Na) Fluorescence Measured by Spectrofluorometry

Figure 3 shows a schematic diagram for the measurement of fluorescence by a nitrogen-pulsed laser spectrofluorometer. A hand-held, single quartz fiber (ϕ = 600 μm, Hamamatsu Photonics, Hamamatsu)

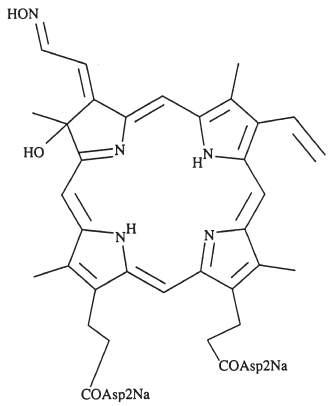

Figure 1. Chemical structure of ATX-S10(Na). Molecular weight = 927.79.

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which was connected to the nitrogen-pulsed laser (LN-103; Photochemical Research Associates, Ontario, Canada), was gently placed in contact with the vascularized cornea. Excitation was accomplished by repetitive 337 nm flashes of 2-nanosecond duration with 5 μW output power (less than 1×10^{-12} J). The fluorescence emitted from the new corneal vessels was passed through the same fiber and was spectrally analyzed by a photomultiplier (R-696; Hamamatsu Photonics). The number of photons was measured using a single photon counting system, with excitation in 2.4 nm steps in the range of 350–800 nm. In order to avoid the direct interference of the excitation light from the photon counting system and simplify the optical system, a dichromatic mirror (reflection at 337 nm) was placed at a 45° angle to the light path (Figure 2).[18–20]

Four rabbits with corneal neovascularization received 12 mg/kg of ATX-S10(Na). Measurements were done before, 5, and 30 minutes, and 1, 2, 4, 6, and 24 hours after dye injection. A different site was selected in the vascularized cornea for each measurement to prevent the adverse effects resulting from the mechanical irritation caused by the fiber contact reported by Schmidt et al.[7] As control, the fluorescence from nonvascularized cornea 2–3 mm below the lower limbus was measured. All measurements were done under topical anesthesia with proparacaine hydrochloride.

The relative fluorescence intensity was obtained by the ratio of 660–680 nm fluorescence to 470 nm fluorescence; the latter was obtained from the biological samples (Figure 3). This index was used to express the accumulation of dye in the previous study of Nakajima et al.[20]

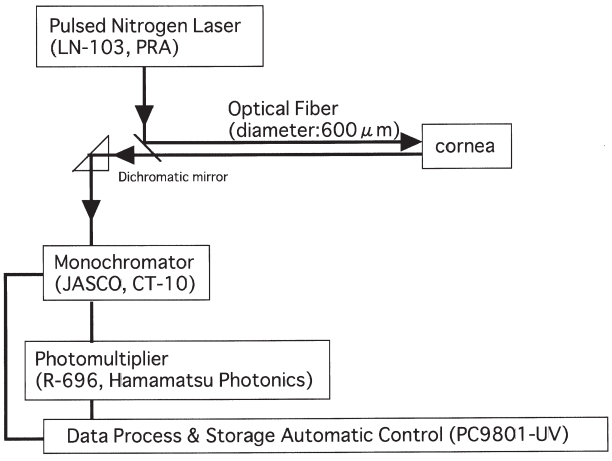

Figure 2. Schematic diagram of equipment for nitrogen-pulsed laser spectrofluorometry.

Localization of ATX-S10 (Na) by Fluorescence Microscopy

ATX-S10(Na) at a dosage of 16 mg/kg body weight was administered through an ear vein in 6 rabbits showing neovascularization. Eyes were enucleated under anesthesia by intravenous pentobarbital (30 mg/kg body weight) at 5 and 30 minutes, and 1, 2, 4, 6, and 24 hours after dye injection. The eyes were fixed in 4% paraformaldehyde solution, embedded in OCT compound (Miles, Elkhart, IN, USA), and frozen in liquid nitrogen. Frozen sections of 5 μm thickness were cut with a cryostat (Jung CM3000; Leica, Nussloch, Germany) and observed under a fluorescence microscope (BX50-FLA; Olympus, Tokyo) coupled to a cooled charged coupling device (CCD) camera (c4880-07; Hamamatsu Photonics) and an image processing system (ARGUS-50/C-CCD; Hamamatsu Photonics) to pinpoint the location of the dye. Sections were excited with 400–440 nm light, and the emission light that passed through a dichroic mirror of 455 nm and a 670–690 nm bandpass filter was detected by the CCD camera. The intensity and exposure time of the excitation light were kept constant throughout the experiments. Frozen sections were stained with hematoxylin and eosin and observed by light microscopy to identify the precise location of fluorescence. As control, 2 eyes from 2 animals with corneal neovascularization that had not received dye injection were used.

Results

Plasma Level of ATX-S10(Na)

The average plasma level of ATX-S10(Na) at 5 minutes after dye injection was 6.79 ± 0.27 μg/mL; there-

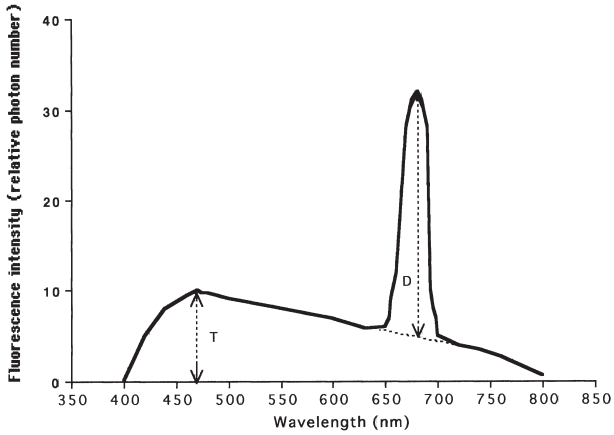

Figure 3. Fluorescence spectrum obtained by nitrogen-pulsed laser spectrofluorometer. T: Fluorescein peak in biological samples usually seen around 470 nm. D: Fluorescence of ATX-S10(Na) at around 680 nm. Ratio (D/T) is used as relative fluorescence intensity of ATX-S10(Na).

after, it decreased rapidly (Figure 4). At 24 hours after dye injection, the average plasma concentration was 0.007 ± 0.007 μg/mL. The half-life calculated from Figure 4 was approximately 13.5 minutes.

ATX-S10 (Na) Fluorescence Measured by Spectrofluorometry

The relative intensity of the fluorescence in the vascularized cornea increased for the first hour, maintained this high intensity for another 3 hours, and then declined (Figure 5). The fluorescence in the nonbascularized cornea increased during the first hour and then quickly declined. The fluorescence in the first hour was always significantly higher than the fluorescence between 1 and 6 hours ($P < .01$, unpaired *t*-test}. The fluorescence values ([C − I]/I; where *C* equals the relative fluorescence intensity of corneal neovascularization and *I* equals the relative fluorescence intensity of nonvascularized cornea) at 1, 2, 4, and 6 hours were 0.95, 1.28, 2.07, and 1.52, respectively.

Localization of ATX-S10 (Na) by Fluorescence Microscopy

At 5 minutes after the injection, fluorescence from ATX-S10(Na) was found both in the lumen of the vascularized cornea and in the surrounding corneal stroma (Figure 6a). Fluorescence in the lumen soon decreased while that in the stroma increased up to 4 hours (Figures 6b,c). Neutrophils infiltrating into the corneal stroma also showed intense fluorescence. Between 2 and 4 hours, fluorescence increased in the walls of the new vessels. Fluorescence diminished in all parts of the cornea by 6 hours, and subsided in all the tissues of the eye by 24 hours (Figure 6d).

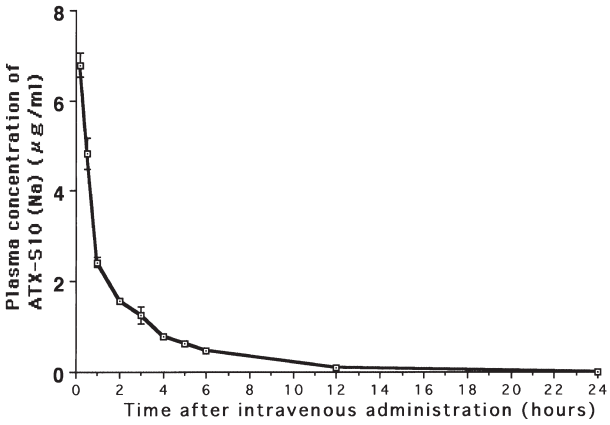

Figure 4. Time-course of plasma concentration of ATX-S10(Na). Mean ± SEM (n = 3 at each time point).

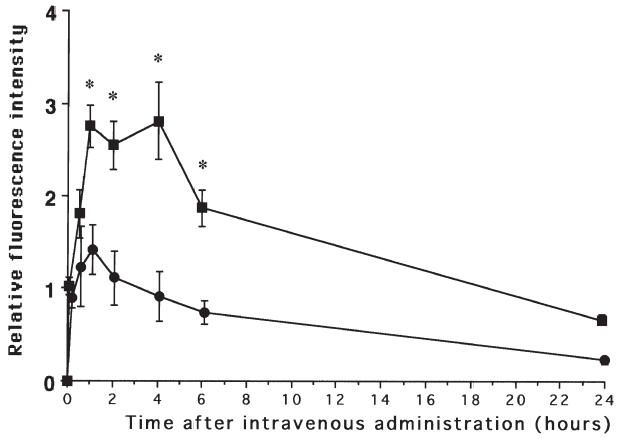

Figure 5. Time-course of relative fluorescence intensity of ATX-S10(Na) (■): ATX-S10(Na) fluorescence detected at site of corneal neovascularization (●): ATX-S10(Na) fluorescence detected at non-vascularized portion of cornea. Mean ± SEM (n = 8). *$P < .01$.

In the iris of animals with neovascularization, fluorescence was found in the lumen of the stromal vessels at 5 minutes after injection. However, there was no marked dye leakage from these vessels (Figure 6e). At 2 hours, fluorescence greatly decreased in the iris vessel walls (Figure 6f), and disappeared by 4 hours (Figure 6g).

In corneas without neovascularization, no fluorescence was observed at any time after dye injection (Figure 6h).

Discussion

The present study demonstrated that ATX-S10(Na) was rapidly eliminated from the body, and this rapid elimination will shorten the stay of patients in a dark room after PDT to prevent skin photosensitization.

Corneal neovascularization that was analyzed by nitrogen-pulsed laser spectrofluorometry covered an area with a diameter of about 500 μm as measured in the photographs of the anterior segments. Since the diameter of the fiber tip was 600 μm, the fluorescence detected by the nitrogen-pulsed laser spectrophotometer came from ATX-S10(Na) in both the plasma and vessel walls of the vascularized cornea and from the corneal stroma within 50 μm around the vascularized area. This recorded fluorescence intensity also included fluorescence from the iris situated below the vascularized area. The fluorescence from the tissue below the iris was negligible since the emission of 337 nm laser light is not readily transmitted to the iris. On the other hand, the fluorescence of

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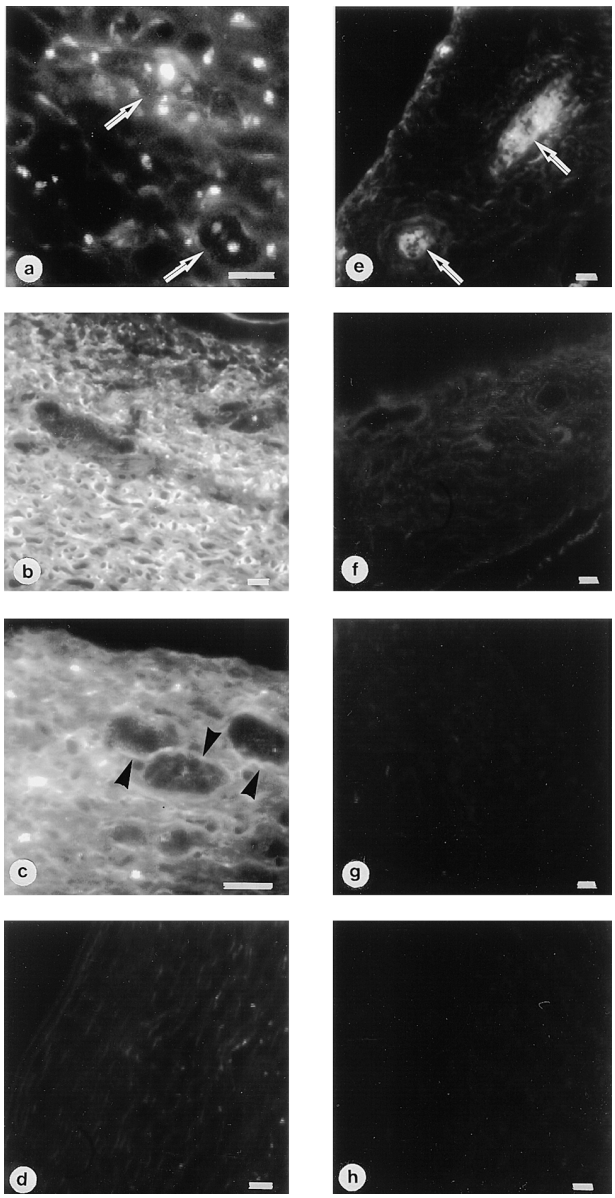

Figure 6. Fluorescence micrographs of sites of corneal neovascularization (a–d), iris neovascularization (e–g) and nonvascularized cornea (h) after injection of ATX-S10(Na). Bar = 45 μm. Cornea: At 5 minutes postinjection (a), fluorescence was present in lumen of neovascularized cornea. (arrow) and adjacent tissues. At 2 to 4 hours postinjection (b,c), fluorescence can be seen in walls of new vessels (arrowhead) and corneal stroma, although fluorescence in lumen of neovascularized cornea has decreased. At 24 hours postinjection (d), no fluorescence is observed. Iris: At 5 minutes postinjection (e), fluorescence can be seen in vessel lumina (arrow), but at 2 hours postinjection (f) weak fluorescence is seen only in walls of vessels. At 4 hours postinjection (g), no fluorescence is observed. Nonvascularized portion of cornea: At 2 hours postinjection (h), no fluorescence is observed.

nonvascularized cornea was thought to be derived from the iris vessels, because there was no fluorescence emitted by the nonvascularized cornea and no dye leakage into the iris stroma from iris vessels that could be detected by fluorescence microscopy.

From the present results obtained by nitrogen-pulsed laser spectrofluorometry, the relative fluorescence intensity of the neovascular cornea at 1, 2, 4, and 6 hours after dye administration was significantly ($P < .01$) higher than that of nonvascularized cornea. Moreover, the fluorescence intensity of the neovascular cornea and surrounding corneal stroma, which was reported to have a value of C − I/I, was highest (2.07) at 4 hours after dye injection. These findings suggest that the difference in the accumulation of ATX-S10(Na) between the neovascular cornea and surrounding normal tissue, ie, the iris situated below the cornea, must be greatest at this time after dye injection.

The fluorescence observed in the walls of new vessels at 2–4 hours after dye injection by fluorescence microscopy was thought to represent the accumulation of ATX-S10(Na) in the endothelial cells of corneal vessels determined as new after a comparison with serial sections from control animals stained with hematoxylin and eosin. In the present fluorescence microscopic study, ATX-S10(Na) uptake by new corneal vessels increased and persisted up to 4 hours, while that by new iris vessels decreased 1 hour after injection. This indicates that the difference in dye accumulation between the vascularized cornea and the vascularized iris became greater between 2 and 4 hours. This accumulation pattern is consistent with the present results obtained by nitrogen-pulsed spectrofluorometry, with our previous fluorescence microscopic observation of choroidal neovascularization,[25] and with our previous ATX-S10(Na)angiographic study on corneal neovascularization.[21]

Marked fluorescence was seen in the corneal stroma between 2–4 hours; however, our previous electron microscopic study[17] demonstrated that no appreciable damage was caused in the collagen fibers and keratocytes in the cornea after PDT, indicating that laser irradiation by PDT was not harmful to the corneal stroma.

The optimal time for the laser irradiation to achieve selective occlusion of new corneal vessels differs among photosensitizers; ie, it is immediately after dye injection for chloroaluminum sulfonated phthalocyanine:[6] 1–2 hours for benzoporphyrin; and 2 or 3 days for hematoporphyrin derivatives. In the case of ATX-S10(Na), we found that it was 2–4 hours after dye injection when the difference in the amount of ATX-S10(Na) in new corneal and iris vessels was the greatest.

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References

1. Dougherty TJ, Kaufman JE, Goldfarb A, Weihaupt KR, Boyle D, Mittleman A. Photoradiation therapy for the treatment of malignant tumors. Cancer Res 1978;38:2628–35.
2. Nelson JS, Liaw LH, Orenstein A, Roberts WG, Berns MW. Mechanism of tumor destruction following photodynamic therapy with hematoporphyrin derivative, chlorin, and phthalocyanine. J Natl Cancer Inst 1988;80:1599–605.
3. Huang AJN, Watson BD, Hernandez ZE, Tseng SCG. Photothrombosis of corneal neovascularization by intravenous rose bengal and argon laser irradiation. Arch Ophthalmol 1989;106:680–5.
4. Corrent G, Roussel TJ, Tseng SCG, Watson BD. Promotion of graft survival by photothrombotic occlusion of corneal neovascularization. Arch Ophthalmol 1989;107:1501–6.
5. Pallikaris IG, Tsilimbaris MK, Iliaki OE, Naoumidi II, Georgiades A, Panagopoulos IA. Effectiveness of corneal neovascularization photothrombosis using phthalocyanine and a diode laser (675 nm). Laser Surg Med 1993;13:197–203.
6. Tsilimbaris MK, Pallikaris IG, Naoumidi II, Vlahonikolis IG, Tsakalof AK, Lydataki SE. Phthalocyanine mediated photodynamic thrombosis of experimental corneal neovascularization: effect of phthalocyanine dose and irradiation onset time on vascular occlusion rate. Laser Surg Med 1994;15:19–31.
7. Schmidt-Erfurth U, Hasan T, Schomacker K, Flotte T, Birngruber R. In vivo uptake of liposomal benzoporphyrin derivative and photothrombosis in experimental corneal neovascularization. Laser Surg Med 1995;17:178–188.
8. Obana A, Gohto Y, Matsumoto M, Miki T. Photodynamic therapy of experimental corneal neovascularization using hematoporphyrin derivatives. J Japan Soc Laser Med 1996;17:31–43.
9. Primbs GB, Casey R, Wamser K, Synder WJ, Crean DH. Photodynamic therapy for corneal neovascularization. Ophthalmic Surg Lasers 1998;29:832–8.
10. Nirankari VS, Baer JC. Corneal argon laser photocoagulation for neovascularization in penetrating keratoplasty. Ophthalmology 1986;93:1304–9.
11. Marsh RJ. Argon laser treatment of lipid keratopathy. Br J Ophthalmol 1988;72:900–4.
12. Baer JC, Foster CS. Corneal laser photocoagulation for treatment of neovascularization. Ophthalmology 1992;99:173–9.
13. Gragoudas ES, Schmidt-Erfurth U, Sickenberg M, et al. Results and preliminary dosimetry of photodynamic therapy for choroidal neovascularization in age-related macular degeneration in a phase I/II study. Invest Ophthalmol Vis Sci 1997;38:S17.
14. Nakajima S, Sakata I, Takemura T, et al. Tumor-localizing and photosensitization of photochlorin ATX-S10. In: Spinelli P, Dal Fante R, Marchesini M, eds. Photodynamic therapy and biomedical lasers. Amsterdam: Elsevier Science, 1992:531–4.
15. Nakajima S, Hayashi H, Sakata I, Takemura T. New photosensitizer—ATX-S10—having type 1 photoreaction. Igaku No Ayumi (J Clin Exp Med) 1993;4:187–8.
16. Gohto Y, Obana A, Miki T, Kaneda K, Sakata I, Nakajima S. Endothelial cell damage of corneal new vessels by photodynamic action with a newly developed chlorin derivative (ATX-S10). Invest Ophthalmol Vis Sci 1995;36:S32.
17. Gohto Y, Obana A, Kaneda K, Miki T. Photodynamic effect of a new photosensitizer ATX-S10 on corneal neovascularization. Exp Eye Res 1998;67:313–22.
18. Kakiuchi Y, Shindo Y, Nakajima S, Koyama T. A new spectrofluorometry as an approach to detect abnormal degeneration of organs. Jpn Soc. ME & BE 1982;20:187.
19. Kakiuchi Y, Shindo Y, Arai T, Koyama T, Nakajima S, Hayashi H. Spectrofluorometric estimation of retained HPD for photoradiation therapy of cancer. Jpn Soc. ME BE 1984; 22:798–9.
20. Nakajima S, Hayashi H, Ornote Y, et al. The tumor-localizing properties of porphyrin derivatives. J Photochem Photobiol 1990;7:189–98.
21. Gohto Y, Obana A, Miki T, Kaneda K, Nakajima S, Takemura T. Time course for the distribution of a photosensitizer (ATX-S 10) to corneal neovascularization. Invest Ophthalmol Vis Sci 1997;38:S513.