

Photodynamic Therapy for Experimental Tumors Using ATX-S10(Na), a Hydrophilic Chlorin Photosensitizer, and Diode Laser

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ATX-S10(Na), a hydrophilic chlorin photosensitizer having an absorption maximum at 670 nm, is a candidate second-generation photosensitizer for use in photodynamic therapy (PDT) for cancer treatment. The effectiveness of PDT using ATX-S10(Na) and a diode laser for experimental tumors was evaluated *in vitro* and *in vivo*. *In-vitro* PDT using ATX-S10(Na) and the diode laser showed drug concentration-, laser dose- and drug exposure time-dependent cytotoxicity to various human and mouse tumor cell lines. In Meth-A sarcoma-implanted mice, optimal PDT conditions were found where tumors were completely eliminated without any toxicity. Against human tumor xenografts in nude mice, the combined use of 5 mg/kg ATX-S10(Na) and 200 J/cm² laser irradiation 3 h after ATX-S10(Na) administration showed excellent anti-tumor activity, and its efficacy was almost the same as that of PDT using 20 mg/kg porfimer sodium and a 100 J/cm² excimer dye laser 48 h after porfimer sodium injection. Microscopic observation of tumor tissues revealed that PDT using ATX-S10(Na) and the diode laser induced congestion, thrombus and degeneration of endothelial cells in tumor vessels, indicating that a vascular shutdown effect plays an important role in the anti-tumor activity of PDT using ATX-S10(Na) and the diode laser.

Key words: Photodynamic therapy — ATX-S10(Na) — Diode laser — Porfimer sodium — Excimer dye laser

In recent years, photodynamic therapy (PDT) utilizing systemic administration of photosensitizer and laser irradiation has attracted considerable interest.¹⁾ This therapy is especially beneficial for preservation of organ functions and for treatment of cancer unsuitable for operation. PDT has also been developed as a treatment for non-cancerous diseases, including ophthalmic disorders, such as choroidal neovascularization,^{2,3)} and cardiovascular disease, such as atherosclerosis.^{4,5)}

Porfimer sodium is the only commercially available photosensitizer applied to the treatment of superficial lung, esophageal, gastric and cervix cancer with combined use of an excimer dye laser.^{6,7)} Although the combination of porfimer sodium and excimer dye laser shows an excellent anti-tumor effect on these tumors, hyperphotosensitivity of skin induced by porfimer sodium forces patients to stay in the dark for 4 weeks after PDT. In order to reduce the hyperphotosensitivity and to enhance the potential of PDT, second-generation photosensitizers are being developed.⁷⁻⁹⁾

ATX-S10(Na), a hydrophilic chlorin photosensitizer synthesized by Toyo Hakka Kogyo Co., Ltd. (Okayama) is

accumulated in tumor tissues and rapidly eliminated from normal tissues within 24–48 h after injection.¹⁰⁻¹³⁾ Moreover, its absorption maximum lies at 670 nm, with a high absorption coefficient of 18500, which is larger than that of porfimer sodium (3000 at 630 nm). A 670-nm laser beam can penetrate deeper into tissues than a 630-nm laser. Therefore, ATX-S10(Na) is an extremely promising candidate as a second-generation photosensitizer of PDT.

In the present study, we evaluated *in vitro* and *in vivo* anti-tumor effects of PDT using ATX-S10(Na) and a 670-nm diode laser, and compared its efficacy with that of PDT using porfimer sodium and an excimer dye laser in human tumor xenograft models.

MATERIALS AND METHODS

Photosensitizers ATX-S10(Na), 13,17-bis(1-carboxypionyl)carbamoylethyl-8-ethenyl-2-hydroxy-3-hydroxyiminoethylidene-2,7,12,18-tetramethylporphyrin sodium salt, was synthesized by Toyo Hakka Kogyo Co., Ltd. Porfimer sodium was obtained from Wyeth Lederle Japan, Ltd. (Tokyo). In the study of *in-vitro* anti-tumor effect, ATX-S10(Na) was dissolved in phosphate-buffered saline (PBS) at the concentration of 10 mg/ml and diluted with culture

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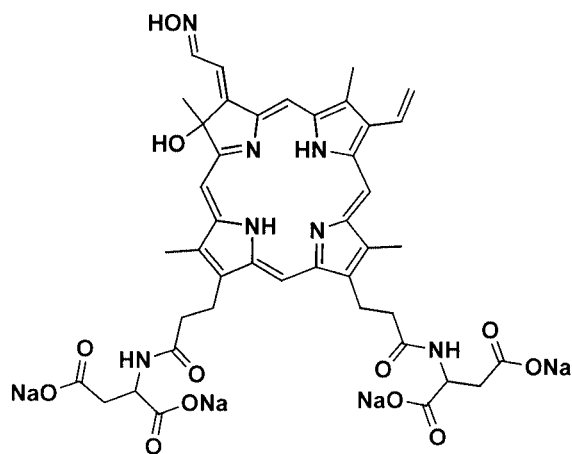


Fig. 1. Chemical structure of ATX-S10(Na).

medium to appropriate concentrations. In the *in-vivo* study, ATX-S10(Na) and porfimer sodium were dissolved in physiological saline and 5% glucose solution for injection, respectively. The chemical structure of ATX-S10(Na) is shown in Fig. 1.

Laser units A diode laser (LD670-05, Hamamatsu Photonics K. K., Hamamatsu) and an excimer dye laser (PDT EDL-1, Hamamatsu Photonics K. K.) were used as light sources for exciting ATX-S10(Na) and porfimer sodium, respectively. The diode laser is a continuous-wave laser operating at 670-nm wavelength. The excimer dye laser is a pulsed laser operating at 630-nm wavelength with the frequency of 40 Hz.

Animals Male BALB/c mice purchased from Charles River Japan, Inc. (Yokohama) were used at 5 weeks of age for implantation of Meth-A sarcoma. Male BALB/c nude mice (BALB/c *nu/nu*) were purchased from Japan SLC, Inc. (Hamamatsu) and implanted with human tumor cells at 7–11 weeks of age. After PDT, animals were maintained in the dark to avoid skin irritation.

Tumors Mouse sarcoma Meth-A cells, mouse colon cancer Colon 26 cells, human esophageal cancer T.Tn cells, human oral cancer KB cells, human cervix cancer HeLa cells and human lung cancer A549 cells were used in this study. T.Tn and KB were purchased from Human Science Research Resource Bank (Osaka), and HeLa was obtained from Riken Cell Bank (Tsukuba). These cells were grown in appropriate medium (Dulbecco's modified Eagle's medium for T.Tn, minimum essential medium with 1% non-essential amino acid for HeLa, KB and A549, RPMI1640 medium for Meth-A and Colon 26) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units/ml benzylpenicillin and 100 μ g/ml streptomycin) in a humidified atmosphere with 5% CO₂ at 37°C.

***In vitro* cytotoxicity test of PDT** In a 96-well microplate, 5×10^3 cells/well of human and mouse cancer cells were incubated with 3.13–50 μ g/ml ATX-S10(Na) at 37°C. The cells were washed with PBS, and irradiated with 25 or 50 J/cm² using a 670-nm diode laser from the underside of the culture plate. After 24-h incubation of cells at 37°C, cell viability was determined by MTS tetrazolium colorimetric assay.¹⁴⁾

Determination of optimum conditions for PDT A single cell suspension of $1-3 \times 10^6$ cells of Meth-A sarcoma was implanted subcutaneously in the right leg of male BALB/c mice. Nine days after tumor implantation, mice were intravenously administered with 3.13–25 mg/kg ATX-S10(Na). Two, four or six hours after injection of ATX-S10(Na), tumor sites were irradiated with a 50–200 J/cm² diode laser under pentobarbital anesthesia. Laser irradiation conditions consisted of irradiation output of 0.4 W and irradiation diameter of 1.5 cm. Mice were observed for 3 weeks after PDT in regard to tumor recurrence and toxicity.

Evaluation of anti-tumor activity in human tumor xenografts in nude mice Single cell suspensions of T.Tn, HeLa and KB cells were implanted subcutaneously in the right leg of male BALB/c nude mice. When the diameter of tumor reached about 4 mm, PDT was performed under the following conditions: 1) no treatment, 2) intravenous administration of 2.5–10 mg/kg ATX-S10(Na) 3 h before irradiation of 200 J/cm² with a diode laser at 670 nm, 3) intravenous administration of 10–20 mg/kg porfimer sodium 48 h before irradiation of 100 J/cm² with an excimer dye laser at 630 nm. Length and width of tumors were measured for 30 days after PDT and tumor volume was calculated by use of the following equation:

$$\text{Tumor volume (mm}^3\text{)} = [\text{Length (mm)}][\text{width (mm)}]^2/2$$

Histological evaluation of PDT BALB/c nude mice that had been subcutaneously implanted with KB cells were intravenously administered with 5 mg/kg ATX-S10(Na). Three hours later, tumor sites were irradiated with 200 J/cm² from a 670-nm diode laser. Immediately after and 1 and 4 h after PDT, under pentobarbital anesthesia, mice were fixed by perfusion from the left ventricle with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Tumor tissues taken from the mice were sectioned at 100- μ m thickness with a microslicer (DTK2000, Dosaka EM Co., Ltd., Kyoto) and stained with hematoxylin-eosin and periodic acid Schiff (PAS) for light microscopic observation. For transmission electron microscopic observation, the tumor samples were postfixed with 1% osmium tetroxide solution, dehydrated in an ethanol series, and embedded in Acracfilm (Nisshin EM Co., Ltd., Tokyo). Ultrathin sections were made, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (TEM; H-7000, Hitachi, Ltd., Tokyo).

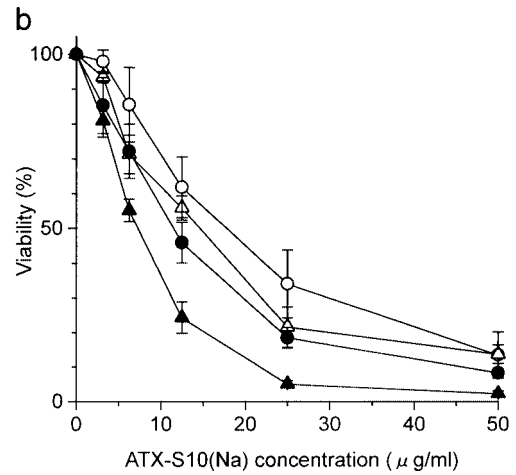
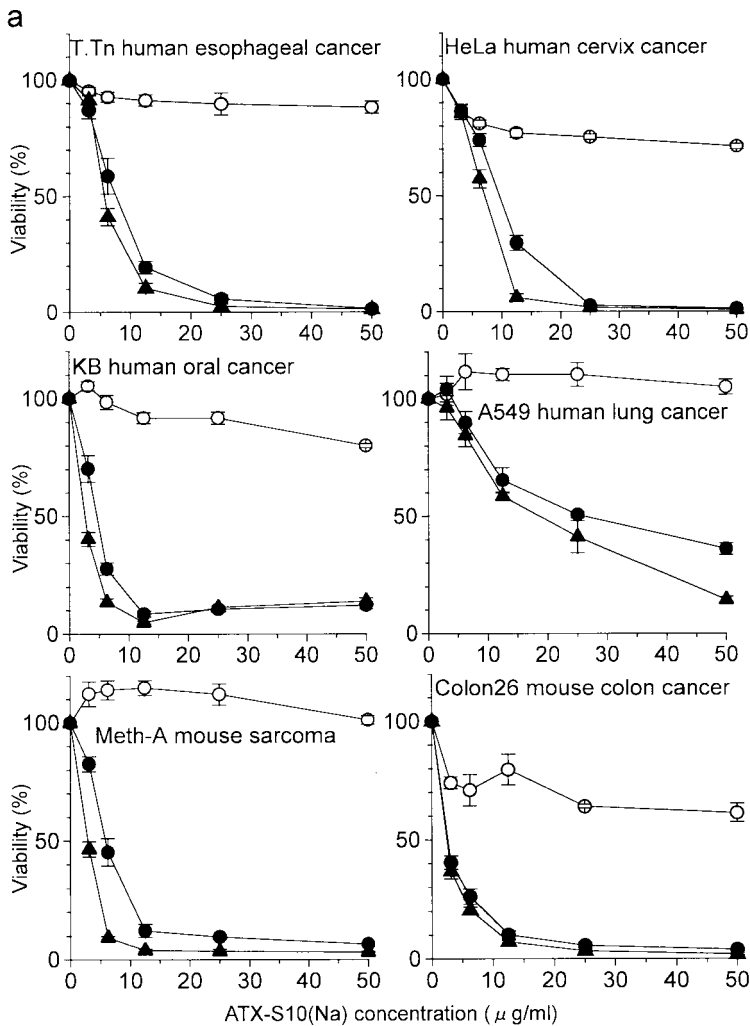


Fig. 2. *In vitro* cytotoxic activity of PDT using ATX-S10(Na) and the diode laser. a) *In vitro* cytotoxicity of PDT in various tumor cell lines. Human and mouse cancer cells were incubated with 3.13–50 $\mu\text{g/ml}$ ATX-S10(Na) at 37°C for 24 h prior to irradiation with 0 (\circ), 25 (\bullet) and 50 (\blacktriangle) J/cm^2 from a 670-nm diode laser. Cell viability was determined by MTS assay. Data represent mean \pm SE ($n=3$). b) Cytotoxicity of PDT at various exposure times after ATX-S10(Na). T.Tn cells were incubated with ATX-S10(Na) at 37°C for 2 h (\circ), 4 h (Δ), 6 h (\bullet) and 24 h (\blacktriangle) prior to irradiation with 50 J/cm^2 from a 670-nm diode laser. Cell viability was determined by MTS assay. Data represent mean \pm SE ($n=3$). Diode laser: 50 J/cm^2 .

RESULTS

***In vitro* cytotoxicity of PDT** The combination of 24-h exposure of cancer cells to ATX-S10(Na) and laser irradiation induced drug concentration- and laser energy-dependent cytotoxicity to various human and mouse cancer cell lines with IC_{50} values of 3–20 $\mu\text{g/ml}$ (Fig. 2a). Cytotoxicity of PDT was also dependent on the exposure time to ATX-S10(Na) in T.Tn cells, and even a 2-h exposure to ATX-S10(Na) could induce sufficient cytotoxic activity *in vitro* (Fig. 2b).

Optimal conditions for PDT *in vivo* Efficacy of PDT is dependent on 3 parameters, i.e. dose of photosensitizer, laser energy and time from ATX-S10(Na) injection to laser irradiation (laser irradiation timing). In order to find optimal parameters for PDT using ATX-S10(Na) and the diode laser, we examined the anti-tumor effect of PDT

Table I. *In vivo* Anti-tumor Activity and Toxicity of PDT under Various Conditions in Meth-A Sarcoma-bearing Mice

Irradiation timing (h)	Laser dose (J/cm^2)	% of tumor-free animals			
		ATX-S10(Na) dose (mg/kg)			
		3.13	6.25	12.5	25
2	50	90	100 ^{a)}	100 ^{a)}	100 ^{b)}
	100	90	100 ^{b)}	90 ^{b)}	10 ^{b)}
	200	100 ^{a)}	100 ^{b)}	20 ^{b)}	0 ^{b)}
4	50	50	50	100 ^{a)}	90 ^{b)}
	100	70	80	100 ^{b)}	70 ^{b)}
	200	80	100 ^{a)}	100 ^{b)}	30 ^{b)}
6	50	40	56	56	89
	100	11	50	100 ^{a)}	60 ^{b)}
	200	50	100 ^{a)}	100 ^{b)}	60 ^{b)}

a) Optimal PDT (100% tumor elimination, no toxicity).

b) Toxic PDT (paralysis/necrosis of irradiation site or death).

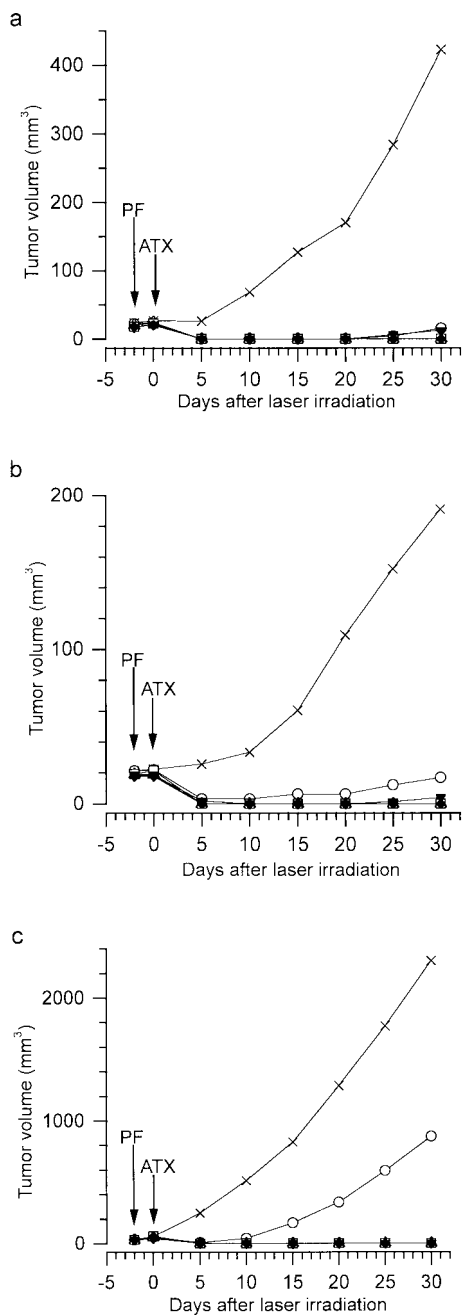


Fig. 3. Anti-tumor effect of PDT in human tumor xenografts in nude mice. HeLa (a), T.Tn (b) and KB (c) cells were implanted subcutaneously into the right leg of nude mice. When the tumor diameter reached about 4 mm, PDT was performed under the following conditions: 1) no treatment (×), 2) 2.5 (○), 5 (△) and 10 (□) mg/kg ATX-S10(Na) plus 200 J/cm² diode laser irradiation at 3 h after ATX-S10(Na) injection, 3) 10 (▼) and 20 (◆) mg/kg porfimer sodium plus 100 J/cm² excimer dye laser irradiation at 48 h after porfimer sodium injection. Tumor size was measured for 30 days after PDT. ATX and PF mean ATX-S10(Na) and porfimer sodium, respectively.

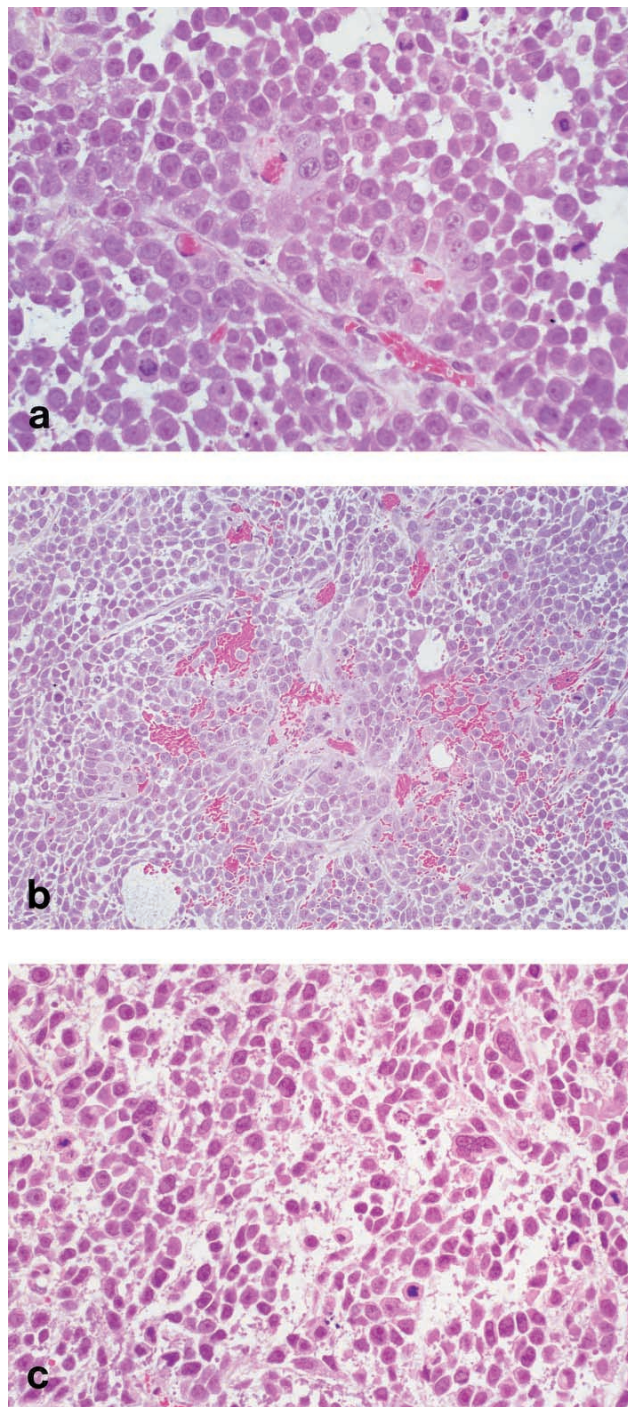


Fig. 4. Light microscopic observation of KB tumor tissues after PDT using ATX-S10(Na) and the diode laser. a) A micrograph obtained immediately after PDT. Congestion and thrombus are found in tumor vessels. b) A micrograph obtained 1 h after PDT. Red blood cells are extravasated from tumor vessels. c) A micrograph obtained 4 h after PDT. Tumor cells are shrunken and necrotic.

under various conditions in tumor-bearing mice. With all of the laser irradiation timings tested, 100% cure without toxicity could be obtained (Table I). When the irradiation timing was set at 2 h, tumors were eliminated in all animals following PDT using 3.13 mg/kg ATX-S10(Na) and 200 J/cm² laser irradiation, 6.25 mg/kg ATX-S10(Na) and 50 J/cm² laser irradiation, or 12.5 mg/kg ATX-S10(Na) and 50 J/cm² laser irradiation with no evidence of adverse effects. With 4-h irradiation timing, PDT using 6.25 mg/kg ATX-S10(Na) and 200 J/cm² laser irradiation and using 12.5 mg/kg ATX-S10(Na) and 50 J/cm² laser irradiation showed complete remission without toxicity. With 6-h irradiation timing, PDT using 6.25 mg/kg ATX-S10(Na) and 200 J/cm² laser irradiation or 12.5 mg/kg ATX-S10(Na) and 100 J/cm² laser irradiation showed complete elimination of tumors without toxicity. When PDT was performed under more severe conditions than those described above (combination of higher dose of ATX-S10(Na) and higher energy of laser irradiation), some animals died or suffered paralysis and necrosis of the right leg, which was the irradiation site. Laser irradiation alone (200 J/cm²) and ATX-S10(Na) administration alone (50 mg/kg) showed no anti-tumor activity and no toxicity in tumor-bearing mice (data not shown).

Anti-tumor effect against human tumor xenografts

Efficacy of PDT using ATX-S10(Na) and the diode laser

in human tumor xenografts was investigated and compared with that of PDT using porfimer sodium and the excimer dye laser. In the 5 mg/kg and 10 mg/kg ATX-S10(Na) groups and the 20 mg/kg porfimer sodium group, tumors were completely eliminated after PDT and recurrence was not observed at the final observation on day 30 (Fig. 3). In the 2.5 mg/kg ATX-S10(Na) group and the 10 mg/kg porfimer sodium group, tumor recurrence was observed. In the 10 mg/kg ATX-S10(Na) group, some animals died 1 or 2 days after PDT.

Histological evaluation The anti-tumor effect of PDT using ATX-S10(Na) and the diode laser was histologically evaluated in KB cell-bearing mice. Mice were treated with 5 mg/kg ATX-S10(Na), and 3 h later, tumors were irradiated with 200 J/cm² from the diode laser. Congestion and thrombus formation were observed in the tumor vessels immediately after laser irradiation, and hemorrhage was observed 1 h after irradiation (Fig. 4, a and b). Necrosis of tumor cells was observed 4 h after irradiation (Fig. 4c). Ultrastructural analysis revealed that endothelial cells were degenerated with swelling of endoplasmic reticulum and mitochondria, cytoplasmic vacuolation, and autolysosome formation just after laser irradiation (Fig. 5).

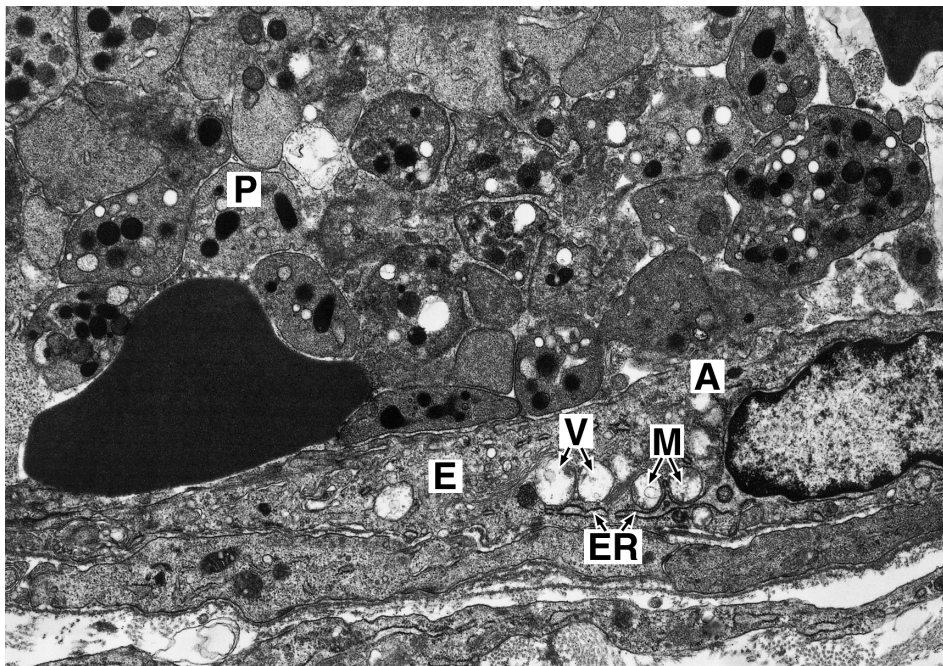


Fig. 5. A transmission electron micrograph obtained immediately after PDT. Endothelial cells (E) are degenerated with swelling of endoplasmic reticulum (ER) and mitochondria (M), cytoplasmic vacuolation (V), and formation of autolysosomes (A). Platelet (P) aggregation is also apparent.

DISCUSSION

In order to evaluate the potential usefulness of PDT using ATX-S10(Na) and the diode laser, we investigated its anti-tumor effect on experimental tumors.

For *in-vivo* PDT using ATX-S10(Na) and the diode laser, we identified some optimal conditions for PDT with 2-, 4- and 6-h irradiation timing. However, in the case of 6-h irradiation timing, the overall healing rate was lower, and a high dose of ATX-S10(Na) was needed to obtain sufficient anti-tumor effect. From the viewpoint of potential clinical application, it was considered preferable to decrease the dose of ATX-S10(Na) and to increase the laser irradiation level as much as possible in order to reduce phototoxicity after PDT and to prevent tumor recurrence owing to insufficient laser irradiation. Therefore, we considered that the best PDT conditions were in the range of 3.13–6.25 mg/kg ATX-S10(Na) and 200 J/cm² laser irradiation at 2–4 h after ATX-S10(Na) injection.

Some animals were damaged in the right leg or died of PDT toxicity. This was probably because the laser beam penetrated the tumor and reached the normal tissues in the right leg, and consequently, severe damage to normal tissues induced systemic shock. In clinical use for humans, however, such acute systemic toxicity of PDT is unlikely to be a problem, because PDT is applied to small superficial tumors whose size is under 1 cm in diameter. ATX-S10(Na) by itself is not toxic; the lethal dose of intravenous ATX-S10(Na) is more than 1000 mg/kg in mice (unpublished data).

The combination of 5 mg/kg ATX-S10(Na) and 200 J/cm² diode laser irradiation at 3 h after ATX-S10(Na) injection showed excellent anti-tumor activity in human xenograft models, and its efficacy was comparable to that of PDT using 20 mg/kg porfimer sodium and 100 J/cm² excimer dye laser irradiation. Since the irradiation timing and laser irradiation wavelength were different in the two PDT procedures, effective doses of ATX-S10(Na) and laser irradiation could not be directly compared. However, the results indicate that PDT using ATX-S10(Na) and the diode laser is likely to be effective against human tumors in clinical use, like PDT using porfimer sodium and the excimer dye laser. The dosage of porfimer sodium used in this study was much higher than that used clinically in humans (2 mg/kg). This was because porfimer sodium is more rapidly eliminated from the body in mice than in humans.

One of the limitations of PDT is tissue penetration by the laser beam. Okunaka *et al.* reported that PDT using a hematoporphyrin derivative and continuous-wave argon dye laser (630 nm) at irradiation doses of 50 and 200 J/cm² could induce tumor necrosis to a depth of 4.1 or 9.4 mm, respectively.¹⁵⁾ Because the wavelength of the diode

laser (670 nm) is longer than that of the argon dye laser, the diode laser beam can penetrate tissues more deeply than the argon dye laser beam. Therefore, we speculate that PDT using ATX-S10(Na) and 200 J/cm² diode laser irradiation may induce tumor necrosis to a depth of about 10 mm.

Like other photosensitizers, photoexcited ATX-S10(Na) produces singlet oxygen,¹⁰⁾ which reacts directly with proteins and lipid in cancer cells, and induces cell-death. On the other hand, histological findings of tumor tissues treated with PDT using ATX-S10(Na) and the diode laser revealed that tumor vessels were damaged soon after PDT, indicating that PDT using ATX-S10(Na) and the diode laser also has an indirect anti-tumor effect that is induced by inhibition of nutritional supply to tumor cells. PDT using porfimer sodium has also been reported to have a vascular shutdown effect, while PDT using disulfonated aluminum phthalocyanine shows little vascular damage.^{16, 17)} Such differences in vascular damage are due to differences in accumulation of dyes in tumor vessels and in the plasma concentration of dyes at the time of irradiation. ATX-S10(Na) is known to accumulate in choroidal neovascularization.²⁾ Therefore, we speculate that ATX-S10(Na) is also accumulated in the newly developed tumor blood vessels. Vascular damage by PDT with porfimer sodium begins to be observed 2 h after laser irradiation,¹⁶⁾ while the damage in the case of PDT of ATX-S10(Na) occurs immediately after laser irradiation. This result indicates that the vascular shutdown effect plays a more important role in the anti-tumor effect of PDT using ATX-S10(Na) than in that of PDT using porfimer sodium.

In conclusion, PDT using ATX-S10(Na) and the diode laser showed an excellent anti-tumor effect on experimental tumors without severe side-effect under optimal conditions, and its efficacy was comparable with that of PDT using porfimer sodium and the excimer dye laser against human tumor xenografts. Therefore, it is expected that this therapy will be clinically useful for the treatment of patients with superficial tumors. Moreover, ATX-S10(Na) appears to be superior to porfimer sodium with respect to phototoxicity. Finally, since the optimal conditions obtained from animal models are not necessarily adequate for clinical treatment of cancer, a comparative pharmacokinetics study between experimental animal and human is needed to identify the optimal conditions for clinical use.

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