Photodynamic Inactivation with Acridine Orange on a Multidrug-resistant Mouse Osteosarcoma Cell Line

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Overcoming multidrug resistance (MDR) is an urgent issue to improve the prognosis of osteosarcoma patients. In this study, we undertook to clarify the effect of photodynamic therapy (PDT) with acridine orange (AO) on the MDR mouse osteosarcoma (MOS/ADR1) cell line, by comparing the outcome with the effect on a chemosensitive osteosarcoma (MOS) cell line. Cultured cells of MOS and MOS/ADR1 cell lines were exposed to AO at various concentrations for various times, followed by long- or short-term (10 or 1 min) illumination with blue light (466.5 nm) for excitation. Living cells were counted by means of the trypan blue exclusion test. The results showed that AO rapidly bound to DNA, RNA and lysosomes of living MOS and MOS/ADR1 cells and also that most tumor cells in both cell lines died rapidly (viability ratio to untreated cells: 1/1000) within 48 h under conditions of continuous or 15-min flash exposure to AO at concentrations above 1.0 μg/ml plus 10-min illumination with blue light. Even after flash exposure to AO at concentrations above 1.0 μg/ml plus 1-min illumination, the viability of MOS/ADR1 cells decreased to a viability ratio of less than 1/1000 within 72 h. Based on these results, we concluded that AO with photoexcitation has a strong cytocidal effect, not only on chemosensitive mouse osteosarcoma cells, but also on MDR mouse osteosarcoma cells. These results suggested that photodynamic therapy with AO may be a new approach to treating MDR human osteosarcomas.

Key words: Multidrug resistance — Osteosarcoma — Acridine orange — Photodynamic therapy

There is no doubt that chemotherapy is the most important treatment for improving the prognosis of patients with osteosarcoma. However, about 30% of these patients have been found to be resistant to chemotherapy.[1–3] Therefore, overcoming multidrug resistance (MDR) is an urgent issue in the management of osteosarcomas. There have been many studies conducted on the modification of MDR in various tumor cell lines,[4–10] but none is clinically applicable at present. Recently, we have found that photodynamic therapy (PDT) with acridine orange (AO) has a strong cytocidal effect on a chemosensitive mouse osteosarcoma cell line. We conducted the present study to clarify the effect of PDT with AO (AO-PDT) on an MDR mouse osteosarcoma cell line, in comparison with the effect on the chemosensitive cell line.

MATERIALS AND METHODS

AO-PDT with mouse osteosarcoma cells   The MDR mouse osteosarcoma cell line (MOS/ADR1)[11] was used in this study. This cell line was established from a radiation-induced mouse osteosarcoma cell line (MOS)[12] by single cell culture after exposure to six-pulsed, stepwise increments of doxorubicin (DOX) concentration ranging from 0.01 to 1 μg/ml. The MOS cells were chemosensitive to most anticancer agents, but the MOS/ADR1 cells showed a classical MDR phenotype with overexpression of P-glycoprotein; they were resistant to DOX, vincristine, vinblastine, etoposide, mitomycin C, and actinomycin D.[11] DOX binding assay demonstrated that more than 90% of the MOS/ADR1 cells were negative for nuclear DOX fluorescence.[11]

We cultured 2×10^5 MOS/ADR1 cells and MOS cells in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C under a 5% CO_2 atmosphere, using 6-well plates. At 24 h, in a preconfluent cell growth condition, the medium of the wells was replaced with 0.025, 0.05, 0.1, 1.0, or 2.0 μg/ml AO containing DMEM. After 15-min exposure to AO, 1400 lx blue light selected through an interference filter (466.5 nm) from a 150-W halogen lamp source (Nikon Fibertrans 2; Nikon, Tokyo) was employed to illuminate the cell surface, to excite AO bound to the cells, using a double fiber tube system (PDT with AO: AO-PDT). This blue light has low energy and does not generate heat. In the continuous AO exposure study, after illumination for 10 min, both tumor cell groups were further cultured in an AO-containing medium. In the flash AO exposure study to minimize the influence of extracellular AO, the tumor cells were cultured in an AO-free medium after 15-min exposure to AO followed by illumination for 10 min. In the short-term

excitation study with flash AO exposure, the tumor cells were illuminated for 1 min after 15-min exposure to AO and were cultured in an AO-free medium. To avoid the influence of room light, all of the procedures were performed in a dark room. The viable cells in each well were counted after 6, 12, 24, 48, and 72 h with a hemocytometer using the trypan blue exclusion test.

We also performed an activated oxygen inhibition test in MOS cells using L-histidine, which is an activated oxygen scavenger.[13] Cells were cultured in the medium containing 50 m*M* L-histidine and 1.0 μg/ml AO for 15 min and then illuminated with blue light for 1 min. Cell viability was measured, after replacement of the medium with AO-free, L-histidine-containing medium.

Morphological changes in mouse osteosarcoma cells after AO-PDT MOS and MOS/ADR1 cells were cultured on a cover slip in each well. After AO-PDT by 15-min exposure to 1.0 μg/ml AO followed by 1-min excitation with blue light, the cells on the cover slip were embedded up side-down on a glass slide and their morphological changes were observed after 0, 6, 12, 48, and 72 h, using a high-resolution fluorescence microscope (Nikon-Microphoto-Fax, Nikon).

Change in DNA ploidy of mouse osteosarcoma cells after AO-PDT Cultured MOS and MOS/ADR1 cells treated with AO-PDT by 15-min exposure to 1.0 μg/ml AO followed by 1-min excitation with blue light were isolated and smeared on glass slides after 0, 6, 12, 48, and 72 h. These cells were fixed with 70% ethanol for 15 min, then stained with propidium iodide (Sigma Chemical Co.,

St. Louis, MO) after 0.5% RNase (Worthington Biochemical Corp., Freehold, NJ). The DNA content of each cell was measured with an epi-illumination type cytofluorometer (Nikon SPM-RFl-D, Nikon) and DNA ploidy was analyzed on a DNA content histogram.[14]

AO-PDT with human fibroblast cell line We investigated the effect of AO-PDT on normal cells, using the human fibroblast cell line, CCD32-SK. The fibroblasts were cultured for 15 min in 1.0 or 2.0 μg/ml AO-containing medium followed by 1-min excitation with blue light, then cell growth was checked.

In vivo **AO exclusion study** To compare AO exclusion from tumor and normal tissue, we performed the following study using an *in vivo* mouse osteosarcoma model.

MOS cells (1×10^6) isolated by trypsinization were inoculated subcutaneously into the back of C3H mice (4-week-old, male; Oriental Bioservice Co., Ltd., Kyoto). After macroscopic tumor formation (8 to 10 mm diameter), mice received an intraperitoneal injection of 10 mg/kg AO, which was found to be the optimum dose for the study in a pilot experiment. At 1, 2, 3, 6, and 12 h, mouse skin covering the tumor was cut open under ether anesthesia, and the tumor and surrounding normal tissue were illuminated with blue light. After taking photographs of each view, including both the tumor and muscle, using a Nikon camera system installed in the stereoscope and a high-resolution color reversal film (Fujichrom, Provia 400; Fujifilm, Tokyo), the difference in fluorescence intensity between the tumor and normal tissue was analyzed quantitatively by NIH Image 1.55 software.
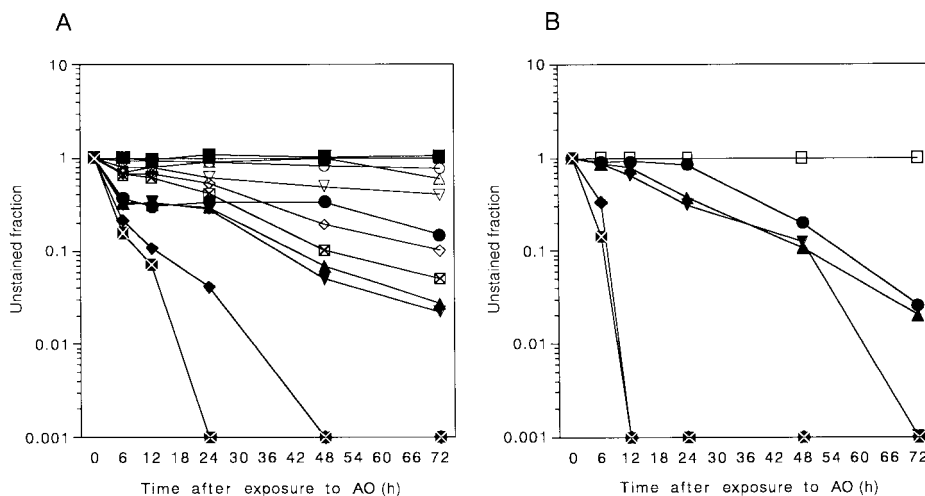

Fig. 1. Tumor cell growth inhibition effect of AO-PDT (continuous exposure to AO). The graphs show the sequential changes in the living tumor cell fraction in MOS (A) and MOS/ADR1 (B) cells treated with continuous exposure to AO at various concentrations plus 10-min illumination with blue light. □ control, ○ 0.025 μg/ml AO, △ 0.05 μg/ml AO, ▽ 0.1 μg/ml AO, ◇ 1.0 μg/ml AO, ⊠ 2.0 μg/ml AO, ■ AO-free-PDT, ● 0.025 μg/ml AO-PDT, ▲ 0.05 μg/ml AO-PDT, ▼ 0.1 μg/ml AO-PDT, ◆ 1.0 μg/ml AO-PDT, ⊗ 2.0 μg/ml AO-PDT.
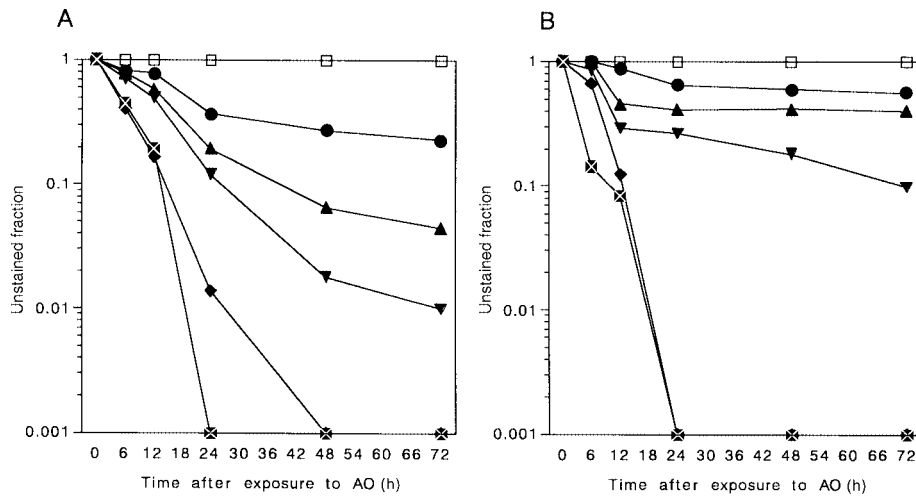
Fig. 2. Tumor cell growth inhibition effect of AO-PDT (flash exposure to AO). The graphs show the sequential changes in the living tumor cell fraction in MOS (A) and MOS/ADR1 (B) cells treated with flash exposure to AO for 15 min followed by 10-min illumination with blue light. □ control, ○ 0.025 μg/ml AO, △ 0.05 μg/ml AO, ▽ 0.1 μg/ml AO, ◇ 1.0 μg/ml AO, ⊠ 2.0 μg/ml AO, ■ AO-free-PDT, ● 0.025 μg/ml AO-PDT, ▲ 0.05 μg/ml AO-PDT, ▼ 0.1 μg/ml AO-PDT, ◆ 1.0 μg/ml AO-PDT, ⊠ 2.0 μg/ml AO-PDT.
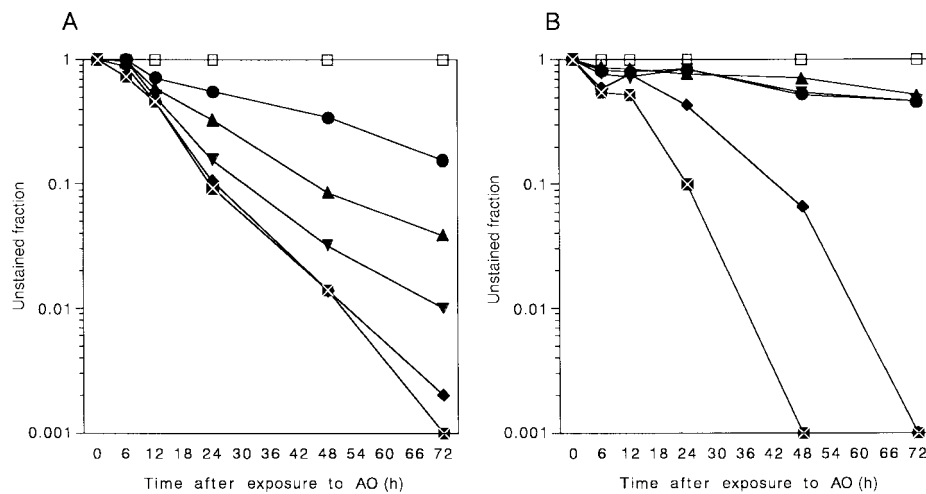

Fig. 3. Tumor cell growth inhibition effect of AO-PDT (short-term illumination). The graphs show the sequential changes in the living tumor cell fraction in MOS (A) and MOS/ADR1 (B) cells with flash exposure to AO for 15 min followed by 1-min illumination with blue light (AO-PDT group). □ control, ○ 0.025 μg/ml AO, △ 0.05 μg/ml AO, ▽ 0.1 μg/ml AO, ◇ 1.0 μg/ml AO, ⊠ 2.0 μg/ml AO, ■ AO-free-PDT, ● 0.025 μg/ml AO-PDT, ▲ 0.05 μg/ml AO-PDT, ▼ 0.1 μg/ml AO-PDT, ◆ 1.0 μg/ml AO-PDT, ⊠ 2.0 μg/ml AO-PDT.

RESULTS

AO-PDT with mouse osteosarcoma cells The viability of MOS and MOS/ADR1 cells continuously exposed to various concentrations of AO decreased dose-dependently (AO group), compared to that of MOS and MOS/ADR1 cells treated without AO exposure or blue light illumina-tion (control group) or with illumination alone (PDT group). However, after 15-min exposure to AO followed by 10-min illumination with blue light (AO-PDT group), the viability of MOS cells, which were continuously exposed to AO at concentrations above 1.0 μg/ml after the illumination with blue light, rapidly (within 48 h) and remarkably (less than 1/1000 viability ratio) decreased

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(Fig. 1A). Under the same conditions, the viability of MOS/ADR1 cells decreased even more rapidly (within 12 h) (Fig. 1B). The viability of MOS or MOS/ADR1 cells cultured in AO-free medium after 15-min flash exposure to AO at concentrations above 1.0 μg/ml with 10-min illumination was also rapidly (within 48 h in MOS cells,

within 24 h in MOS/ADR1 cells) and remarkably (1/ 1000 of viability ratio) decreased dose-dependently (Fig. 2, A and B). Even after 1-min illumination with blue light follwing 15-min exposure to AO at concentrations above 1.0 μg/ml, the viability of MOS or MOS/ ADR1 cells was decreased to a viability ratio of less than 2/1000 within 72 h for MOS cells (Fig. 3A), and less than 1/1000 within 72 h for in MOS/ADR1 cells (Fig. 3B).

In the activated oxygen inhibition test with L-histidine, the viability of MOS cells exposed to 1.0 μg/ml of AO for 15 min followed by 1-min illumination with blue light in a medium containing 50 mM L-histidine was not decreased, compared to that of non-treated cells or cells treated with L-histidine alone (Fig. 4).

Morphological changes in mouse osteosarcoma cells after AO-PDT Both of the MOS and MOS/ADR1 cell types emitted green fluorescence from the cytoplasm and nucleus under a fluorescence microscope with blue excitation at 0 h after 15-min exposure to 1.0 μg/ml of AO followed by 1-min illumination with blue light. This green fluorescence was due to AO binding to RNA and probably also to DNA. Perinuclear particles emitted orange fluorescence which derived from condensed AO bound to lysosomes. After 24 h, both the cytoplasm and nucleus of each cell became swollen, and green fluorescence was weaker, while orange fluorescence became more intense and widely spread in the cytoplasm. After 72 h, most of the cells died with a ruptured cell membrane (Fig. 5).
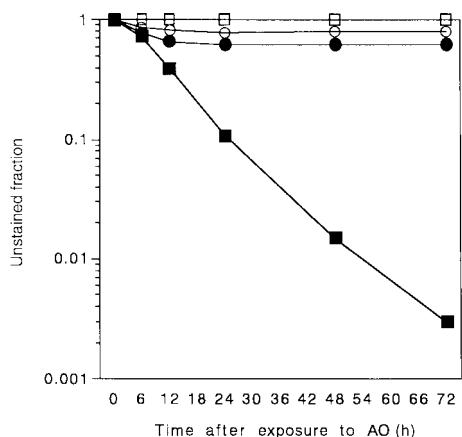

Fig. 4. Active oxygen-scavenging effect of L-histidine. The graph shows the sequential changes in the living MOS tumor cell fraction under flash exposure to 1.0 μg/ml AO for 15 min plus 1-min illumination with blue light in the presence of 50 mM L-histidine in the medium. □ control, ○ L-histidine, ■ AO-PDT, ● AO-PDT+L-histidine.
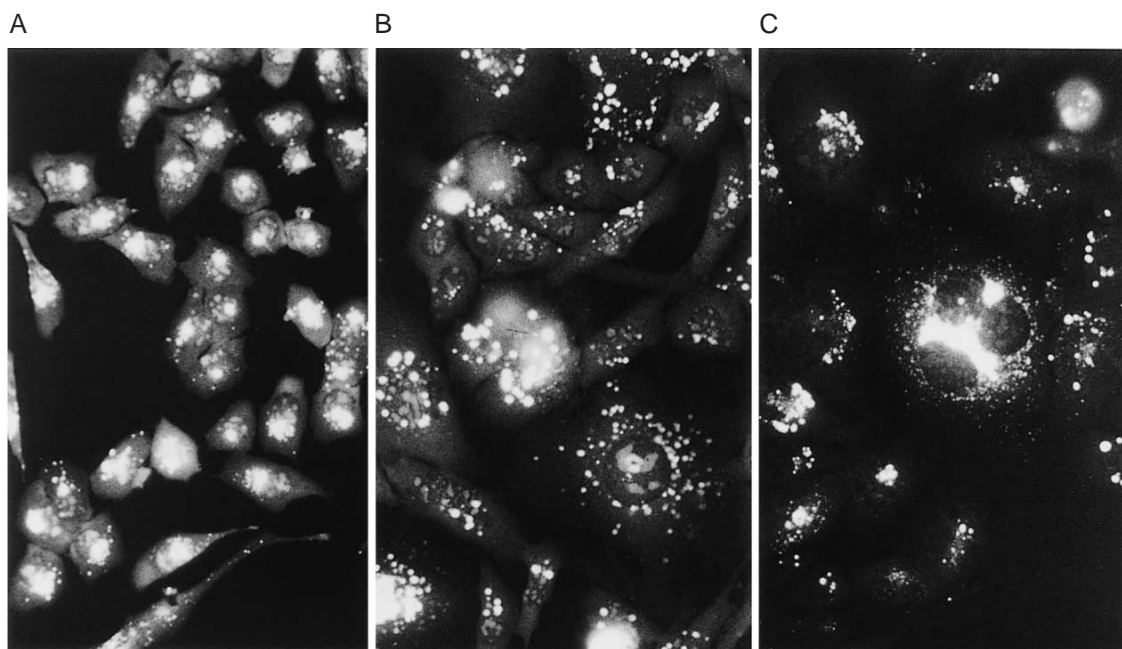

Fig. 5. Morphological changes of MOS/ADR1 cells after AO-PDT. Fluorescence photomicrographs show the sequential changes in morphology of the MOS/ADR1 cells after 15-min exposure to 1.0 μg/ml AO followed by 1-min excitation with blue light. A, at 0 h; B, at 24 h; C, at 72 h.
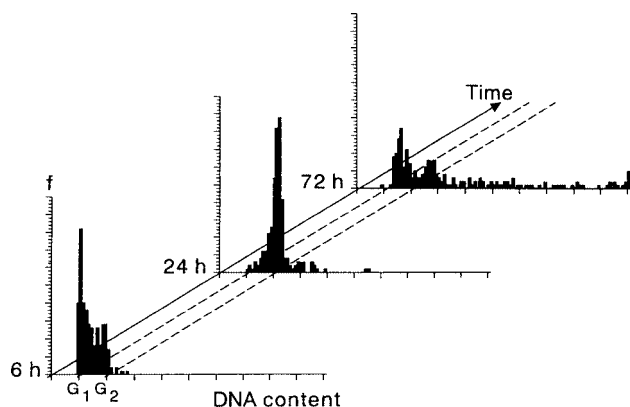
Fig. 6. DNA ploidy analysis of MOS/ADR1 cells after AO-PDT. The graph shows the sequential changes in DNA ploidy in the MOS/ADR1 cells at 0, 24, and 72 h after 15-min exposure to 1.0 μg/ml AO followed by 1-min excitation with blue light.
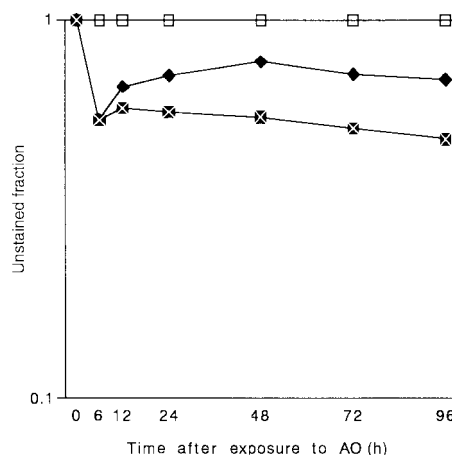

Fig. 7. Cell growth inhibition effect of AO-PDT on normal fibroblasts. The graph shows the sequential changes in the living cell fraction in the human fibroblast cell line CCD32-SK after 15-min exposure to 1.0 or 2.0 μg/ml AO followed by 1-min excitation with blue light. □ control, ◆ 1.0 μg/ml AO-PDT, ⊠ 2.0 μg/ml AO-PDT.

Change in DNA ploidy of mouse osteosarcoma cells after AO-PDT The MOS cells or MOS/ADR1 cells consisted of many hypotetraploid cells (G_1-phase) with their S- and G_2-phase cells before AO-PDT. However, at 24 h after 15-min exposure to 1.0 μg/ml of AO followed by 1-min illumination with blue light, DNA ploidy showed that there were many G_2-phase arrested cells. After 72 h, most cells had a polyploid DNA content greater than octaploidy (Fig. 6).

AO-PDT with human fibroblast cell line The viability of human fibroblast CCD32-SK cells, which were exposed to 1.0 and 2.0 μg/ml AO for 15 min followed by 1-min illumination with blue light was not significantly decreased, compared to non-treated cells even after 96 h (Fig. 7).

In vivo **AO exclusion study** Fig. 8 shows the time courses of change in the AO fluorescence intensity in the tumor and normal tissue after AO injection, analyzed by NIH Image. The fluorescence intensity from the tumor and normal tissue increased quickly within 1 h, but at 2 h, that of normal tissue decreased more rapidly than that of the tumor. AO fluorescence from the tumor gradually decreased until 12 h and was maintained at a higher level than that of normal tissue.

DISCUSSION

It is well-known that some sarcomas are chemoresistant. For instance, 30% of osteosarcomas are considered to be multidrug-resistant.[1–3, 15, 16] In order to improve the prognosis of patients with osteosarcoma, overcoming chemoresistance is most important. Experimentally, many candidate MDR-modifying agents have been found,[4–10] but only a few have clinical potential, for example, vera-
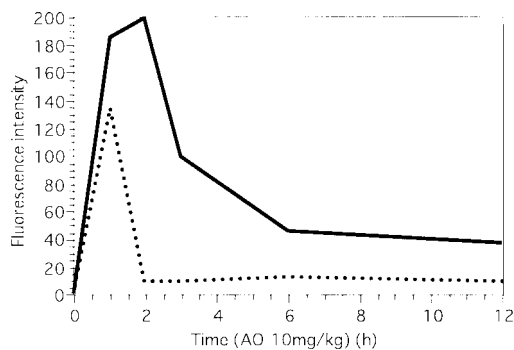

Fig. 8. *In vivo* AO exclusion from osteosarcoma and normal tissue. The graph demonstrates the sequential changes in fluorescence intensity of the MOS tumor and surrounding normal tissue after intraperitoneal injection of 10 mg/kg AO by NIH Image analysis. —— tumor, ---- normal tissue.

pamil,[4] FK506[5] and MS-209.[6, 8] Those agents can modify the MDR associated with P-glycoprotein,[15–18] but not other drug resistances associated with the MRP/GS-X pump or glutathione.[19, 20]

Recently, we have found that AO with PDT has a strong cytocidal effect on the chemosensitive mouse osteosarcoma cell line, MOS. There have been several reports to demonstrate the effect of AO on MDR tumor cells. One report showed that anti-tumor acridine compounds, such as proflavine (which is biologically different from AO), exhibited cross resistance with the P388/ADR cell line, which exhibits MDR associated with P-glycoprotein, and this resistance was modified by verapamil.[21] Another study

demonstrated that there was no difference in AO accumulation between sensitive and multidrug-resistant K562 cells, but resistant cells had significantly higher red fluorescence than sensitive cells.[22] Therefore, it is of interest to clarify the effect of PDT with AO on the MDR mouse osteosarcoma cell line, MOS/ADR1, which expresses P-glycoprotein.

The results of our present study demonstrate that AO rapidly entered MOS and MOS/ADR1 cells, and bound to DNA,[23, 24] RNA[25, 26] and lysosomes.[27] These cells were rapidly killed by PDT with 15-min exposure to 1.0 μg/ml AO followed by 1-min illumination with blue light. Therefore, it is evident that AO is not excluded rapidly from the cells by P-glycoprotein, unlike DOX. There have been no reports on any cell lines resistant to AO, but as we showed in the *in vivo* study, AO accumulated in mouse osteosarcoma was retained longer than in surrounding normal tissues, including muscle, fibrous tissue or adipose tissue. Furthermore, we also demonstrated that cultured normal fibroblasts were not completely killed by AO-PDT. These results suggest that normal cells can exclude AO more rapidly than osteosarcomas. We stained 18 fresh human sarcomas, including osteosarcomas, liposarcomas, and malignant fibrous histiocytomas, with AO (1 μg/ml) and found that all sarcomas emitted intensive AO fluorescence, while surrounding normal tissues did not.[28] Therefore, normal cells are probably resistant to AO. The difference in sensitivity to AO between normal cells and tumor cells may be based on mechanisms such as the following: normal cells may have a pump-out system, different from P-glycoprotein, to eliminate AO to the outside through the cell membrane, or tumor cells may have a larger amount of nucleic acid, especially transfer or ribosomal RNA, which is an AO attack site, compared to normal cells, or AO may bind more tightly to the tumorous RNA.

The PDT with AO caused rapid and remarkable cell death. This result is consistent with the fidings in experimental tumor models reported previously.[29–31] Cells died with cell membrane rupture after cytoplasmic swelling, as shown in Fig. 5. The mechanism of the cytocidal effect by AO-PDT in mammalian cells was reported to be that AO excited with blue light emits fluorescence and generates singlet oxygen ($^1O_2$), which is produced by energy transfer from stacked triplet AO to triplet oxygen ($^3O_2$) (type II photochemical reaction), and the singlet oxygen peroxidizes lipid of the cell membrane.[26] The study involving flash exposure to AO indicated that extracellular AO in the medium did not cause a cytocidal effect, so that singlet oxygen generated by intracellular AO bound to RNA, DNA or lysosomes may be important in AO-PDT. The results of the activated oxygen inhibition test in this study showed that the cytocidal effect of AO-PDT on mouse osteosarcoma cells was completely inhibited in medium containing L-histidine, which is an activated oxygen scavenger.[13] At the nuclear DNA level, cell growth was arrested at the G_2 phase, then DNA synthesis without nuclear and cytoplasmic division occurred, leading progressively to polyploidy. This polyploidization was consistent with the morphological change of nuclear enlargement, as shown in Fig. 5. In our experience, a dose of AO alone higher than 1.0 μg/ml led to tumor growth inhibition with polyploidization (data not shown), but the inhibition ratio was much lower than with AO-PDT. Therefore, polyploidization occurring in AO-PDT may be caused mainly by enhancement of the original anti-tumor effect of AO after excitation. Blue light of 466.5 nm used in the study has neither a cytocidal nor a heat generation effect, because the energy is very low compared to usual laser beam energy commonly employed in PDT for cancer. In this excitation system, we can evaluate the pure AO-PDT effect on tumor cells without the influence of other energy factors.

Based on these results, we conclude that AO may not show cross resistance to MDR associated with P-glycoprotein, and that AO-PDT has a strong cytocidal effect on MDR osteosarcoma cells as well as chemosensitive osteosarcoma cells. Accordingly, we believe that AO-PDT may become a useful technique for MDR human osteosarcomas.

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