Acridine Orange Inhibits Pulmonary Metastasis of Mouse Osteosarcoma

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Abstract. *Although the survival of patients with osteosarcoma has improved following development of chemotherapy and surgery, the presence of pulmonary metastases indicate a poor prognosis. We developed photodynamic and radiodynamic therapies with acridine orange (AO-PDT and AO-RDT) for minimally invasive surgery to treat musculoskeletal sarcomas and reported a good clinical outcome of local control and limb function. We investigated the effect of AO-PDT using flash-wave light (FWL) on pulmonary metastasis of mouse osteosarcoma. In in vitro and in vivo studies, AO alone and AO-PDT significantly inhibited cell invasion and the growth of pulmonary metastases from primary mouse osteosarcoma. AO may have a specific metastasis-inhibitory effect, different from the effect of AO-PDT. The fluorovisualization effect on pulmonary metastases following intravenous AO administration showed that pulmonary metastases localized on the lung surface were recognized as brilliant green lesions. In conclusion, AO-PDT using FWL inhibited cell invasion and pulmonary metastases in mouse osteosarcoma; therefore, this treatment modality might be applicable for treating pulmonary metastasis from malignant musculoskeletal tumors in humans.*

The survival of patients with osteosarcoma has improved due to the development of chemotherapy and surgical techniques (1); however, approximately 15-20% of osteosarcoma patients undergo detectable metastatic disease (2-4). Primary metastases of osteosarcoma affect the lung in 87% of the cases, distant bones in 21% of the cases, and other soft tissues in 9% of the cases, thus result in poor prognosis (5).

We developed photodynamic and radiodynamic therapies with acridine orange (AO-PDT and AO-RDT) as a minimally invasive surgery for treating musculoskeletal sarcomas, making it possible to preserve excellent limb function with a low risk of local tumor recurrence. On the basis of the satisfactory outcome of clinical trials involving more than 100 patients with high-grade malignant bone and soft tissue sarcomas, our previous reports revealed that this modality was clinically applicable (6-11). Nevertheless, in those clinical trials, AO was locally administered by flooding of the surgical field after tumor resection, and prognosis of these patients was better than that of patients treated with conventional wide tumor resection. Therefore, AO-PDT may inhibit metastasis. Furthermore, our previous studies demonstrated that intravenous AO administration had excellent fluorovisualization effect for photodynamic diagnosis (PDD) of mouse osteosarcoma (12) and that AO-PDT with intravenous AO injection significantly inhibited tumor growth of mouse osteosarcoma (13). Moreover, we have previously reported that a high-power flash-wave light (FWL) from a xenon lamp in AO-PDT exerts a stronger cytocidal effect than a continuous-wave light (CWL) on a mouse osteosarcoma cell line (14, 15).

In this study, we investigated whether PDT using FWL with intravenous AO administration exhibits anti-metastatic activity in the development of pulmonary metastasis in mouse osteosarcoma.

Materials and Methods

Tumor cell line and cell culture. The mouse osteosarcoma cell line derived from Dunn's osteosarcoma, LM8, which is highly metastatic, was used in the present study (16). LM8 cells were harvested in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum at 37˚C in 5% CO_2. All experiments described below were started after 24 h of cell culture.

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Light sources. A xenon lamp was used as the source of FWL (17). The illumination machine, High Power Strobo Flash XF-1000 (Nissin Electric Co., Kyoto, Japan), was used for FWL irradiation. The light irradiation frequency of the FWL was 30 Hz and the pulse width was less than 1 ms. The energy generated by one shot-irradiation with FWL was 0.1 J cm^2, and the illuminance level was 10^6 lux.

In vitro study. Cell invasion assay: LM8 cells were divided into six groups (n=6): group 1, exposure to AO-free DMEM and no FWL irradiation (control group (C)); group 2, exposure to AO-free DMEM and irradiation with FWL for 10 min (irradiation group (IR)); groups 3-6, exposure to different concentrations (0.1 and 1.0 μg/ml) of AO (Sigma-Aldrich Munich, Germany) for 10 min and no FWL irradiation (group 3, 0.1-AO; group 4, 1.0-AO; group 5, 0.1-AO+IR; group 6, 1.0-AO+IR). At the beginning of the treatment, the medium in each group was exchanged with DMEM containing different concentrations of AO (0.1 and 1.0 μg/ml) or with AO-free DMEM. After exposure to AO for 10 min, the medium was washed out to remove the AO, and AO-free DMEM was added. The cells were isolated from the culture dishes by trypsinization and loaded into the upper chamber in growth medium-containing 6-well BD Matrigel™ Invasion Chamber, pore size, 8 μm (BD Bioscience, CA, USA) at a cell density of 5×10^4 cells per well and were excited with FWL for 10 min. After this treatment, the cells were harvested for 24 h at 37˚C in 5% CO_2, following which, duplicate membranes of the chambers were processed and stained with HE. Invading cells were evaluated by counting the number of cells in all fields under high-power light microscopy.

In vivo study. Mouse osteosarcoma model: A suspension containing 1×10^6 cells isolated from culture dishes using trypsinization was inoculated into the soft tissues, including the subcutaneous tissue and muscles of the back, after removal of the hair at the implantation site in C3H mice (5-week-old males) (Japan SLC. Inc., Shizuoka, Japan). Subsequent experiments were conducted on tumors that grew to a macroscopically detectable size (3-6 mm in diameter) within 10 days.

Inhibition of pulmonary metastasis by AO-PDT using FWL: Tumor-bearing mice were divided randomly into four groups of five mice each: group 1, no treatment (C); group 2, irradiation with FWL alone for 10 min (IR); group 3, intravenous administration of AO at 1.0 mg/kg alone (AO); and group 4, intravenous administration of AO at 1.0 mg/kg followed by irradiation with FWL for 10 min (AO+IR). Tumor-bearing mice administered AO *via* the tail vein were exposed to FWL illumination for 10 min at 2 h after AO injection, as previously described (13). Briefly, AO mice administered were placed in a stainless steel bowl under anesthesia induced by intraperitoneally administered pentobarbital sodium and were exposed to FWL irradiation using an illumination machine (XF-1000) for 10 min. AO was used at a concentration of 1.0 mg/kg because our previous studies showed that this concentration yields the strongest cytocidal effect and the lowest toxicity in mice (12, 13). The irradiation time (10 min) was also determined on the basis of the results of previous studies (12, 13, 15, 18, 19). On day 28 after the above treatment, AO was administered intravenously at a concentration of 1.0 mg/kg to tumor-bearing mice *via* the tail vein. After 2 h, mice in each group were sacrificed under anesthesia and the lungs were removed. The lungs were illuminated using a 5,000-luminance blue light selected through an interference filter (450-490 nm) from a 500-W high-power xenon lamp source (SAN-EI Electric MFG Co., Ltd., Tokyo, Japan) and guided through a single fiber tube. The fluorescence emitted from the AO accumulated in the pulmonary metastases was detected using a digital camera system C-5050 equipped with an absorption filter (>520 nm) set at a distance of 10 cm from the tumor surface (photodynamic diagnosis with acridine orange (AO-PDD)). This procedure was conducted in a dark room (12). Image data from the photogram were entered into a personal computer using Adobe Photoshop 7.0 software (Adobe Systems Co., MD, USA). The number of metastatic lesions on the surface of the lungs was counted on the AO-PDD images, regardless of the tumor size (18). The removed lungs were fixed in 10% formalin and embedded in paraffin. The paraffin-embedded lung tissues were cut at their maximum dimensions and were stained with HE. The number of pulmonary metastases was then counted based on histological findings, regardless of the tumor size, under high-powered light microscopy.

Statistical analysis. Statistical analysis was performed using the StatView statistical software version 5.0 (SAS Institute Inc Cary, NC, USA). Significant differences among the groups were evaluated using Student's *t*-test. *p*-Values less than 0.05 were considered statistically significant.

All experiments were performed in accordance with the guidelines in the Declaration of Helsinki and the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education.

Results

In vitro study. Cell invasion assay: Figure 1 shows the results of the study involving the BD Matrigel™ Invasion Chamber and demonstrates that the average number of LM8 cells that had passed through the chamber membrane after 24 h was 114±14 in group 1 (C) and 117±12 in group 2 (IR). There were no significant differences between these groups. In the AO group alone, the average numbers of invading cells were 61±19 in group 3 (0.1-AO) and 61±6 in group 4 (1.0-AO). The ability of cells to pass through the membrane was significantly inhibited in the AO group than in the control group ($p<0.01$). In the AO-PDT group, the number of invading cells was 34±19 in group 5 (0.1-AO+IR) and 5±1 in group 6 (1.0-AO+IR). In both groups, there was a remarkable decrease of the invading number of cells compared to groups 1 (C) and 2 (IR) ($p<0.01$). In the AO-PDT group, the inhibitory effect of AO on cell invasion was concentration dependent.

In vivo study. Count of pulmonary metastatic lesions by histology and fluorescence imaging after AO administration: Figure 2 shows the brilliant green fluorescence emitted from the *ex vivo* pulmonary metastatic lesions localized on the lung surface in group 1 (C) under blue light excitation of AO selectively bound to tumor cells. Visualization of the pulmonary metastases on fluorescence images (right side of the figure, fluorovisualization) is much easier than macroscopic detection under normal light (left side of the figure). Figure 3 shows the histological findings of the pulmonary metastases

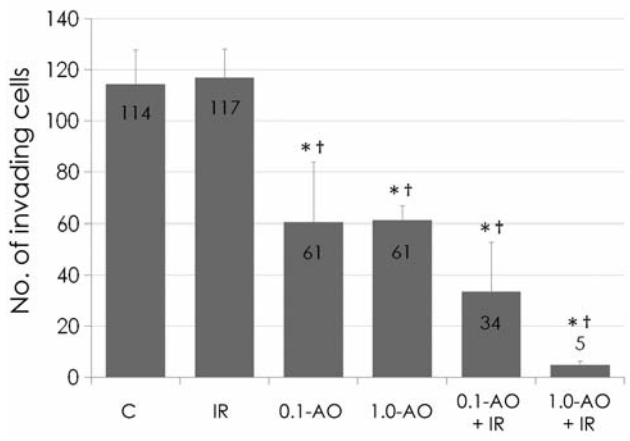

Figure 1. *Number of invading LM8 cells after acridine orange (AO) exposure or followed by illumination with flash-wave light (FWL) in the BD Matrigel™ Invasion Chamber assay. C, control group; IR, FWL irradiation group; 0.1-AO, exposure to 0.1 μg/ml of AO group; 1.0-AO, exposure to 1.0 μg/ml of AO group; 0.1-AO+IR, exposure to 0.1 μg/mL of AO and FWL irradiation group; 1.0-AO+IR, exposure to 1.0 μg/mL of AO and FWL irradiation group. *, p<0.01 vs. C; †, p<0.01 vs. IR.*

originating from the osteosarcoma in group 1 (C). Not only were surface metastatic lesions seen on fluorescence images but deeply located lesions were detectable as well. The graph in Figure 4 presents both the numbers of the pulmonary metastatic lesions counted on histological sections and the surface metastatic lesions visualized using AO fluorescence images in each group. There was no significant differences in the number of lesions detected with each method in groups 1 (C), 2 (IR), and 4 (AO+IR); however, in group 3 (AO), twice the number of lesions was observed on fluorescence images as compared to the ones detected by histological examination. Numerous pulmonary metastases were detected in groups 1 (C) and 2 (IR), whereas in group 4 (AO+IR), a markedly reduced number of metastases was detected (C *vs*. AO+IR: histological examination, $p<0.04$ and fluorescence imaging, $p<0.04$; IR *vs*. AO+IR: histological examination, $p<0.03$ and fluorescence imaging, $p<0.01$). Group 3 (AO) also exhibited a significant decrease in the number of metastatic lesions as compared to groups 1 (C) and 2 (IR) (C *vs*. AO: histological examination, $p<0.05$ and fluorescence imaging, $p<0.05$; IR *vs*. AO: histological examination, $p<0.05$ and fluorescence imaging, $p<0.05$).

Discussion

Patients with osteosarcoma commonly undergo hematogenous metastasis to the lungs (3, 20). The prognosis of patients with pulmonary metastasis remains poor despite multimodal treatments, including chemotherapy and surgery (3, 5). Recently, the efficacy of percutaneous radiofrequency ablation in treating pulmonary metastases arising from sarcoma (21) was reported; however, there are presently few effective therapies available for treating multiple pulmonary metastases from osteosarcoma.

We developed and established a limb salvage surgery combined with minimal invasive tumor excision and AO-PDT followed by AO-RDT to preserve excellent limb function with a low risk of local tumor recurrence (6-11). In clinical studies, AO was locally administered by flooding of the surgical field after tumor resection, but the prognosis of patients was better than that of patients treated with conventional wide tumor resection surgery. Therefore, we speculated that AO-PDT may inhibit metastasis.

The results of this study revealed that AO-PDT using FWL and AO alone, had a remarkable *in vitro* anti-invasive effect and an *in vivo* anti-metastatic effect on mouse osteosarcoma cells. Since previous studies reported that AO-PDT has a strong cytocidal effect on malignant musculoskeletal sarcomas (14, 15, 19), cell apoptosis by AO-PDT may reduce cellular invasive ability. Our *in vivo* study demonstrated that AO-PDT using FWL as the excitation light after intravenous AO injection, was also highly effective for inhibiting the growth of pulmonary metastases from primary mouse osteosarcomas, without resection of the local lesion, thus suggesting that AO-PDT prevents metastasis to the lung. This effect may be primarily due to growth inhibition of the primary tumor; however, cellular pulmonary metastasis is considered to occur within 10 days after tumor cell inoculation in our osteosarcoma model (22) because mice in which the primary tumor had been widely resected later died of pulmonary metastasis without local recurrence. Therefore, a possible mechanism underlying on the pulmonary metastasis-inhibitory effect of AO-PDT may be the destruction of cellular metastatic lesions by AO or AO-PDT. Since mice treated using AO-PDT received illumination over the entire body, the high-power FWL penetrated the chest wall to exert a cytocidal effect against metastatic lesions incorporating intravenously administered AO.

Interestingly and unexpectedly, cell invasion was significantly suppressed in the group exposed to AO alone *in vitro*. Inhibition of pulmonary metastases was also observed in mice treated with AO alone *in vivo*. It has been established that AO alone, does not have a strong cytocidal effect against mouse osteosarcomas either *in vitro* or *in vivo* (13, 15); therefore, the mechanism of this effect remains unclear. AO may have a specific metastasis-inhibitory effect, which in different from the effect of AO-PDT. High-grade malignant tumors exist in an acidic environment, which is considered to accelerate metastasis (23). AO selectively binds to acidic structures in the tumor cells, such as lysosomes or acidic vesicles containing a large amount of protons (low pH), in order to neutralize the acidic conditions (24). The lysosome contains many enzymes, some of which

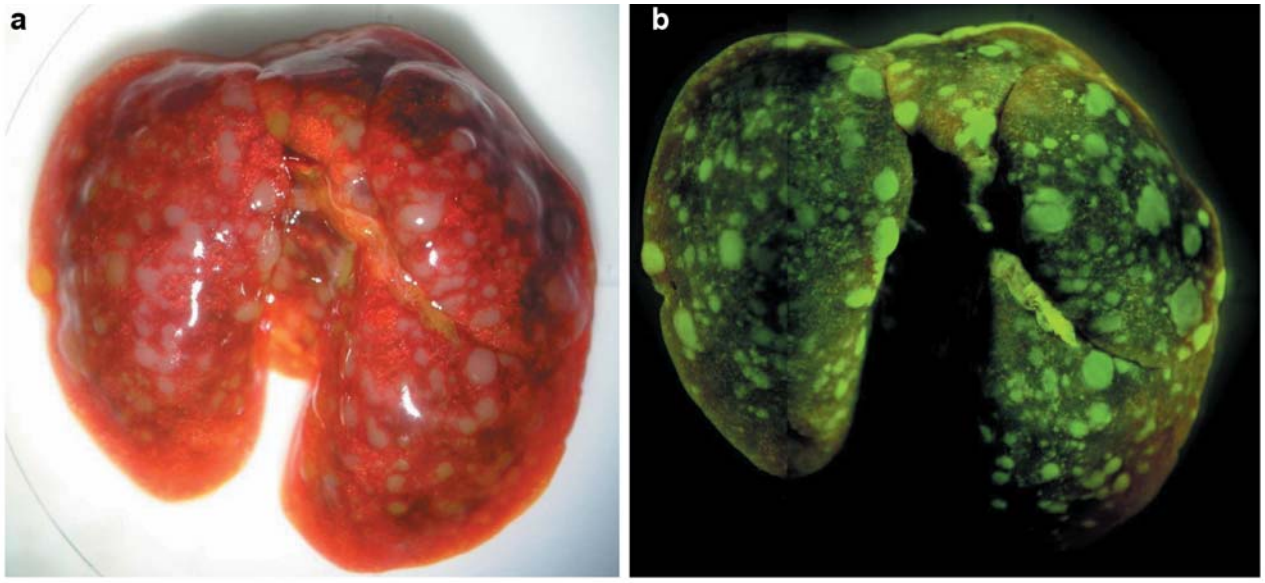

Figure 2. *Stereoscopic findings of pulmonary metastatic lesions localized on the lung surface under ordinary light (a) and under blue light using a yellow absorption filter (b).*

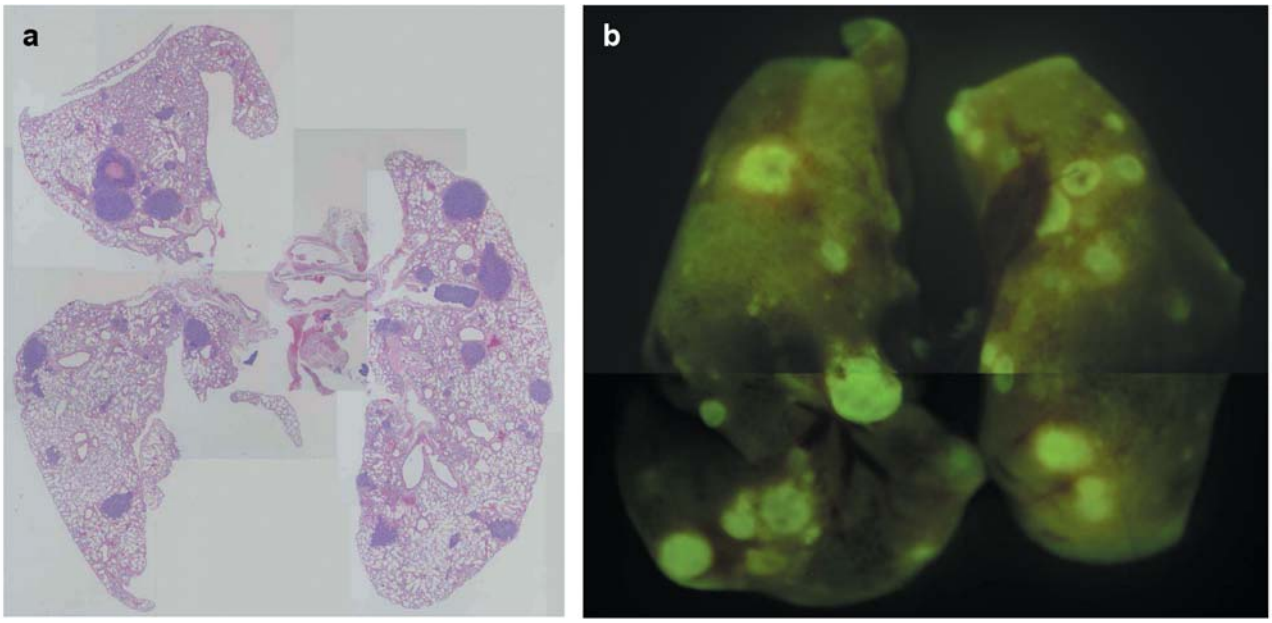

Figure 3. *Histological findings (a) and fluorescence images in photodynamic diagnosis with acridine orange (AO-PDD) (b) of pulmonary metastatic tumors from mouse osteosarcoma.*

are important for tumor invasion and metastasis, such as cathepsin groups, metalloproteases, and heparinases (25-27). AO may induce dysfunction of lysosomal enzymes related to pH. This may be one of the mechanisms underlying the prevention of pulmonary metastasis by AO, although further investigation is needed. Another potential mechanism is that after PDT, antitumor immunity may be enhanced, and tumor antibodies may kill pulmonary metastatic tumor cells (28). Recently, it was reported that exosomes induced by PDT also enhance antitumor immunity after stimulating macrophage antigen expression (29). These tumor immunity-related mechanisms which suppress pulmonary metastasis of osteosarcoma are fascinating in the clinical application of AO-PDT.

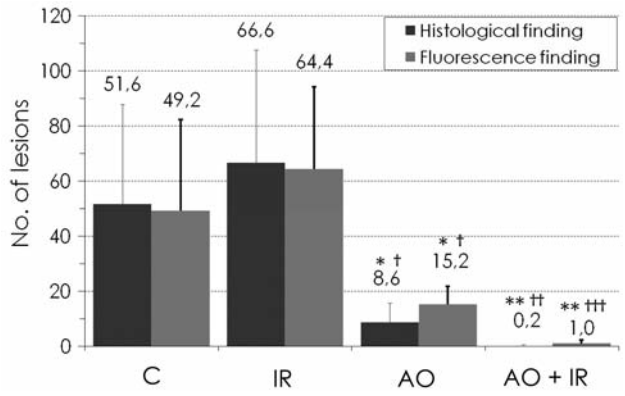

Figure 4. *Number of pulmonary metastases detected using histological and fluorescence imaging methods in each group studied with acridine (AO) alone and photodynamic therapy with acridine orange (AO-PDT). C, control group; IR, flash-wave light (FWL) irradiation group; AO, intravenous administration of 1.0 μg/ml of AO group; AO+IR, intravenous administration of 1.0 μg/ml of AO and FWL irradiation group. *, p<0.05 vs. C; †, p<0.05 vs. IR; **, p<0.04 vs. C; ††, p<0.03 vs. IR; †††, p<0.01 vs. IR.*

An *ex vivo* fluorescence imaging study of the fluorovisualization effect on pulmonary metastases following intravenous AO administration showed that pulmonary metastases localized on the lung surface were recognized as brilliant green lesions. Clinically, this effect may be very useful for determining the surgical margin during endoscopic resections, such as thoracoscopy, bronchoscopy, cystoscopy, and laparoscopy (30-32). Although many experimental methods have been suggested for assessing pulmonary metastases (33, 34), fluorescent detection following intravenous AO administration, referred to as AO-PDD, is useful and easier than other methods. If the fluorescence intensity of AO over the entire lung was measured using a photomultiplier, more accurate estimation of the metastatic tumor volume, as compared with that using the count method, could be possible.

In conclusion, AO-PDT using FWL inhibited cell invasion and pulmonary metastasis in mouse osteosarcoma; therefore, we believe that this treatment modality may be applicable for treating pulmonary metastasis of malignant musculoskeletal tumors in humans, although additional studies are needed to verify this finding.

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