Flash Wave Light Strongly Enhanced the Cytocidal Effect of Photodynamic Therapy with Acridine Orange on a Mouse Osteosarcoma Cell Line

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Abstract. *A photodynamic therapy technique with acridine orange (AO-PDT) was experimentally developed and applied clinically to musculoskeletal sarcoma patients to reduce the surgical margin and obtain good limb function. Furthermore, various modalities to enhance and strengthen the cytocidal effect of AO-PDT were investigated. A recent report revealed that the use of stronger unfiltered xenon light in AO-PDT enhanced the cytocidal efficacy of this treatment modality. Therefore, in this study, we investigated whether the use of a flash wave light (FWL) from a xenon lamp, as compared to that of the conventional continuous wave light (CWL), might enhance the cytocidal effect of AO-PDT, using the mouse osteosarcoma cell line, LM8. For an equal energy dose (79.6 joules/cm^2), AO-PDT using FWL (10 minutes excitation) was found to exert a significantly stronger cytocidal effect than that using CWL (18 seconds excitation). For the same excitation time (10 minutes' excitation), the use of FWL (79.6 joules/cm^2) was associated with a significantly stronger cytocidal effect of AO-PDT than that of CWL (3,820 joules/cm^2). These results reveal that the use of FWL entails the need for a lower excitation energy and shorter excitation time than that of CWL for the cytocidal effect of AO-PDT to be observed against the osteosarcoma cells. In addition, FWL also has the advantage of generating low heat and of having the ability to homogenously illuminate a wider area. We therefore concluded that FWL is more useful for AO-PDT than CWL in terms of saving on the excitation time and of obtaining good efficacy of destruction of the residual tumor in the treatment of musculoskeletal sarcomas.*

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We previously demonstrated that the combination of photodynamic therapy (PDT) and radiodynamic therapy (RDT) with acridine orange (AO-PDT and AO-RDT) is useful for preventing local tumor recurrence after surgical resection with an intra-lesional or marginal surgical margin in patients with high-grade malignant musculoskeletal sarcomas (1-3). In AO-PDT, a high-power xenon lamp is used as the light source to excite AO, which in turn induces the formation of reactive oxygen species from the intracytoplasmic oxygen to exert its cytocidal effect. It has been suggested that the use of a stronger light source may enhance this cytocidal effect. A recent report from another group demonstrated a stronger cytocidal effect of AO-PDT against mouse osteosarcoma cells with the use of an unfiltered higher-illuminance (lux) xenon light as the light source (4).

The flash wave light (FWL) xenon lamp, which is commonly used in photography in the dark, has a high illuminance with low heat, as compared to that of the continuous wave light (CWL) xenon lamp which has conventionally been used for AO-PDT. Therefore, in this study, we undertook to investigate whether the use of FWL, as compared to that of CWL for AO-PDT, might enhance the cytocidal activity of this treatment modality against a mouse osteosarcoma cell line.

Materials and Methods

Tumor cell line. The mouse osteosarcoma cell line derived from Dunn's osteosarcoma, LM8, which possesses strong metastatic ability, was used for the study (5). The LM8 cells were plated in a 96-well plate per treatment group at a cell density of 1x10^4 cells per well and harvested in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37˚C in a 5% CO_2 atmosphere. All the experiments shown below were started after 24 hours of cell culture.

Light source. A xenon lamp source was used to obtain both the FWL and CWL used in this study. The illumination machines KFS-30HJ (Ushio Electric Inc., Tokyo, Japan) and XEF-501S (SAN-EI Electric MFG. Co., Ltd., Tokyo, Japan) were used, respectively, to

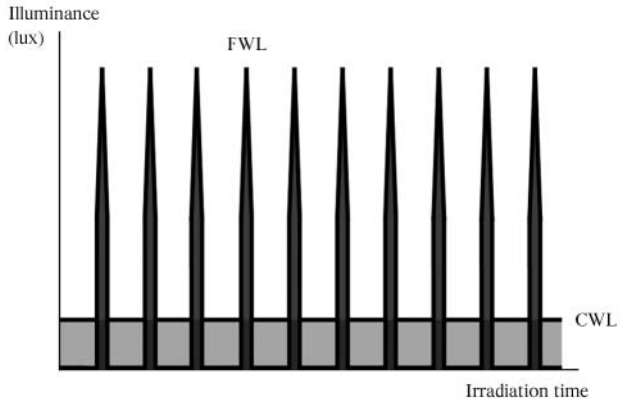

Figure 1. *Schema showing the difference in the illuminance and irradiation time between CWL and FWL.*

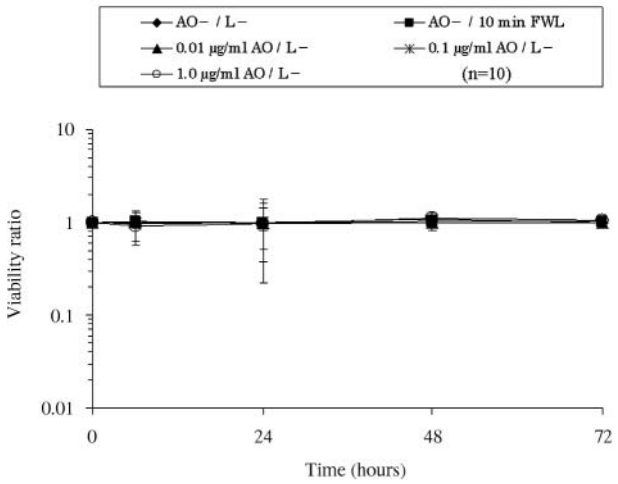

Figure 2. *Sequential changes in the viability of the LM8 cell fractions in the control groups.*

obtain the FWL and CWL. The light illumination frequency of the FWL was 60Hz and the pulse width was less than 1 millisecond (Figure 1). The energy generated by 1 shot-illumination with FWL was 15 joules. The illuminance of the CWL is 10,000 lux, whereas that of the FWL is 1,000,000 lux.

Control groups. The control group was divided into five subgroups: Group 1: exposure to AO-free DMEM and no light excitation (AO– / L–); Group 2: exposure to AO-free DMEM and excitation with FWL for 10 min (AO– / 10 min FWL); Groups 3-5: exposure to different concentrations (0.01, 0.1 and 1.0 μg/ml) of AO (Sigma-Aldrich Chemie Gmbh, Germany: Lot No. 122K0522) for 10 min and no light excitation (group 3: 0.01 μg/ml AO / L–, group 4: 0.1 μg/ml AO / L–, group 5: 1.0 μg/ml AO / L–).

The viability ratio of the LM8 cells (viability of each well divided by that of the cells in the AO– / L– well) in each well was assessed at 6, 24, 48 and 72 hours after excitation using the 3-(4,5-dimetylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay, which measures the mitochondrial dehydrogenase activity, in accordance with the manufacturer's instructions (Promega Corporation, USA).

Comparative study of the cytocidal effect of AO-PDT administered using FWL and CWL for an equal energy dose. At the beginning of the treatment (0 hour), the medium in each well was exchanged with DMEM containing different concentrations of AO (0.01, 0.1 and 1.0 μg/ml), or with AO-free DMEM for the control. The LM8 cells were excited with FWL for 10 min or CWL for 18 s, to expose the cells to an equal energy dose (79.6 J/cm^2) (0.01 μg/ml AO / 10 min FWL, 0.1 μg/ml AO / 10 min FWL, 1.0 μg/ml AO / 10 min FWL, and 0.01 μg/ml AO / 18 s CWL, 0.1 μg/ml AO / 18 s CWL, 1.0 μg/ml AO / 18 s CWL). After the excitation, the medium in each well was washed out to remove AO and replenished with AO-free DMEM. The viability ratio of the LM8 cells in each well was assessed at 6, 24, 48 and 72 hours after the irradiation by the MTS assay.

Comparative study of the cytocidal effect of AO-PDT administered using FWL and CWL for an equal excitation time. At the beginning of the treatment (0 hour), the medium in each well was exchanged with DMEM containing different concentrations of AO (0.01, 0.1 and 1.0 μg/ml), or with AO-free DMEM for the control. The LM8 cells were excited with FWL (79.6 J/cm^2) or CWL (3,820 J/cm^2) for 10 min (0.01 μg/ml AO / 10 min FWL, 0.1 μg/ml AO / 10 min FWL, 1.0 μg/ml AO / 10 min FWL, and 0.01 μg/ml AO / 10 min CWL, 0.1 μg/ml AO / 10 min CWL, 1.0 μg/ml AO / 10 min CWL). After excitation, the medium in each well was washed out to remove AO and replenished with AO-free DMEM. The viability ratio of the LM8 cells in each well was assessed at 6, 24, 48 and 72 hours after the irradiation by MTS assay.

Statistical analysis. Statistical analysis was performed using the StatView statistical software (version 5.0; SAS Institute Inc. Cary, NC, USA). Significant differences between the various groups were evaluated using Student's *t*-test. *P*-value less than 0.05 was considered to be significant.

Results

Viability ratio of the LM8 cells in the control subgroups. The viability ratio of the LM8 cells did not differ significantly at any of the time-points examined among the 5 control subgroups, namely, AO– / L–, AO– / 10 min FWL, 0.01 μg/ml AO / L–, 0.1 μg/ml AO / L–, and 1.0 μg/ml AO / L– (Figure 2). AO at these concentrations was not cytotoxic in the absence of light excitation. Furthermore, irradiation with FWL or CWL without AO was also not cytotoxic.

Cytocidal effect of AO-PDT administered using FWL and CWL for an equal energy dose. The LM8 viability of the 0.01 μg/ml AO / 10 min FWL group was significantly lower than at any time-point after end of treatment, compared with the group of 0.01 μg/ml AO / 18s CWL ($p<0.001$) (Figure 3). At 48 and 72 hours after the end of treatment, the viability ratio of the LM8 cells exposed to 0.1 and 1.0 μg/ml of AO showed no significant difference between the groups excited

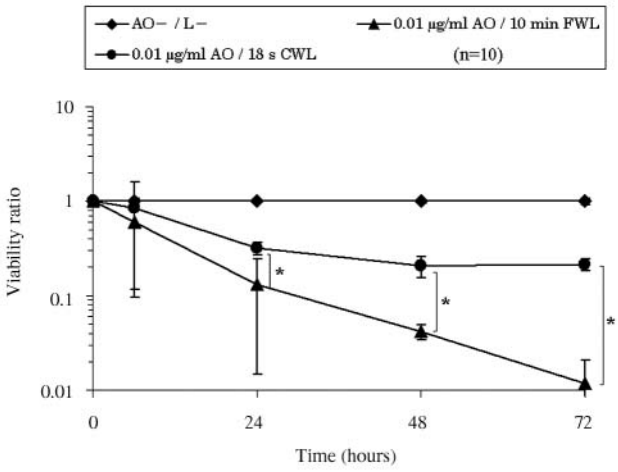

Figure 3. *Sequential changes in the viability of the LM8 cell fractions subjected to AO-PDT (AO concentration of 0.01 μg/ml) using FWL or CWL at an equal energy dose. *p<0.001.*

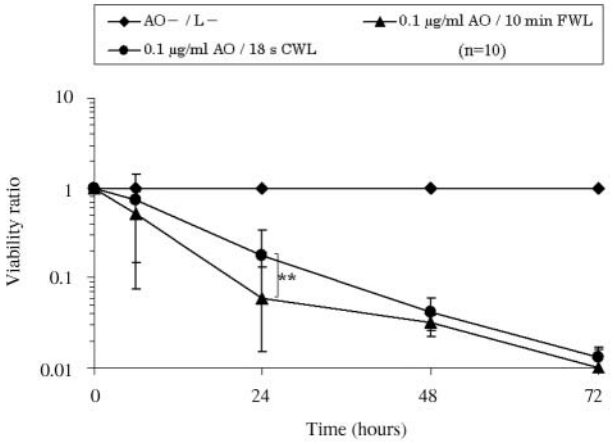

Figure 4. *Sequential changes in the viability of the LM8 cell fractions subjected to AO-PDT (AO concentration of 0.1 μg/ml) using FWL or CWL at an equal energy dose. **p<0.05.*

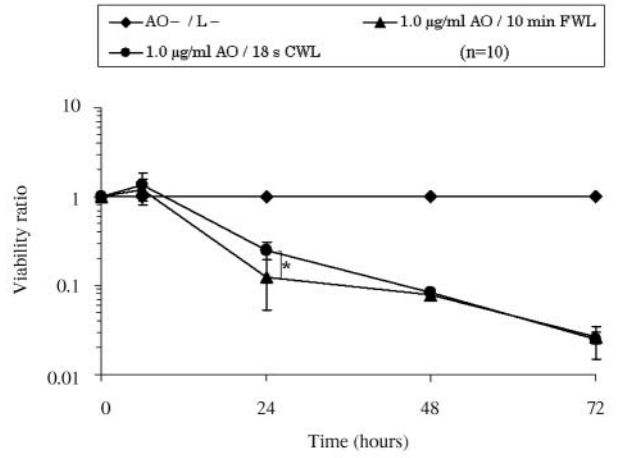

Figure 5. *Sequential changes in the viability of the LM8 cell fractions subjected to AO-PDT (AO concentration of 1.0 μg/ml) using FWL or CWL at an equal energy dose. *p<0.001.*

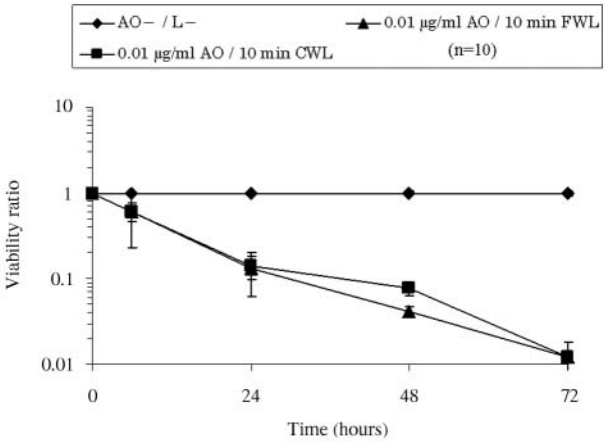

Figure 6. *Sequential changes in the viability of the LM8 cell fractions subjected to AO-PDT (AO concentration of 0.01 μg/ml) using FWL or CWL for an equal excitation time.*

with FWL for 10 min and CWL for 18 s, whereas at 24 hours, the viability ratio of the cells exposed to 0.1 and 1.0 μg/ml of AO excited by FWL for 10 min was significantly lower than that of cells excited by CWL for 18 s ($p<0.05$, $p<0.001$) (Figures 4 and 5).

Cytocidal effect of AO-PDT administered using FWL and CWL for an equal excitation time. There was no significant difference in the viability ratio of the LM8 cells between the 0.01 μg/ml AO / 10 min FWL and 0.01 μg/ml AO / 10 min CWL groups until 72 hours after the end of treatment (Figure 6). Although there was no significant difference in the viability ratio of the LM8 cells between the 0.1 μg/ml AO / 10 min FWL and 0.1 μg/ml AO / 10 min CWL groups at 72 hours, the viability ratio of the cells in the 0.1 μg/ml- AO / 10min- FWL group was significantly lower at 24 and 48 hours, as compared with that in the 0.1 μg/ml AO / 10 min CWL group ($p<0.04$) (Figure 7). The viability ratio of the cells in the 1.0 μg/ml AO / 10 min FWL group was also significantly lower at 24 and 48 hours, as compared with that in the 1.0 μg/ml AO / 10 min CWL group ($p<0.001$) (Figure 8).

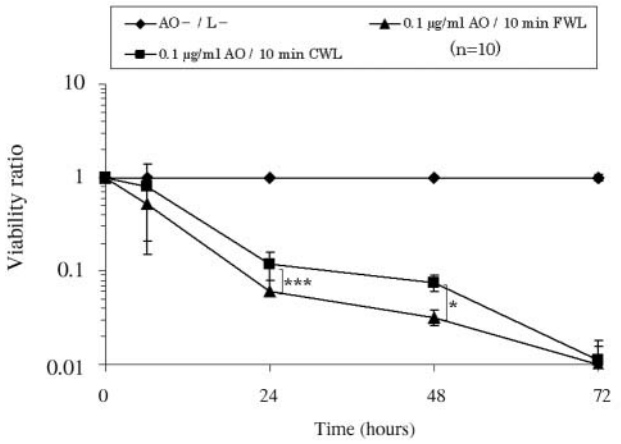

Figure 7. *Sequential changes in the viability of the LM8 cell fractions subjected to AO-PDT (AO concentration of 0.1 μg/ml) using FWL or CWL for an equal excitation time. *p<0.001, ***p<0.04.*

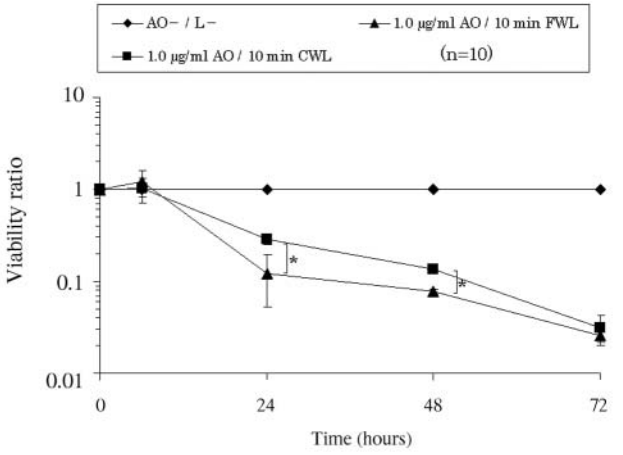

Figure 8. *Sequential changes in the viability of the LM8 cell fractions subjected to AO-PDT (AO concentration of 1.0 μg/ml) using FWL or CWL for an equal excitation time. *p<0.001.*

Discussion

Each photosensitizer used for photodynamic therapy has a specific maximum absorption wavelength of photons at which it is most effectively excited. For example, porphyrin, which is commonly used as a photosensitizer in PDT for many epithelial cancers, such as cancer of the skin (6, 7), bladder (8, 9), lung (7, 10, 11), esophagus (7, 12, 13), bile duct (14, 15), breast (16, 17), and brain (18, 19), has a maximum absorption wavelength of 630 nm; therefore, laser with a wavelength of 630 nm is used for the excitation of porphyrin. AO also has a maximum absorption wavelength of 492 nm (blue color) and emits green (533 nm) and red (656 nm) fluorescence. The green fluorescence arises from the monomeric form of AO binding to RNA or DNA, while the red fluorescence arises from the aggregated form of AO binding to acidic vesicles such as lysosomes (20-24).

For the excitation of AO in clinically applied AO-PDT for the treatment of musculoskeletal sarcomas, we conventionally use blue light with a wavelength of 460-500 nm, selected through a xenon lamp with an interference filter because homogeneous illumination of blue light over a wide area is necessary for the fluorovisualization effect of AO (1-3) as well as for the strong cytocidal effect of AO-PDT on remnant tumor cells which are spread extensively throughout the surgical field because of curettage. A laser beam, while having a high energy, illuminates only a very narrow area of the tumor (less than 1 cm) (25, 26), therefore it is not suitable for such large-sized tumors as musculoskeletal sarcomas. Even laser beam scanning of such tumors takes time. On the other hand, a xenon lamp can illuminate a wide area of the tumor in one shot and the illumination range can be easily changed, allowing a strong cytocidal effect of the excited AO. Even in economic terms, it is much cheaper than a laser (27).

In the fluorovisualization of musculoskeletal sarcomas, blue-light excitation is the most powerful for obtaining strong fluorescence emission of AO (28-31), however, there was no evidence to suggest that the use of the maximum absorption wavelength always induces the maximum cytocidal effect in AO-PDT. A recent study from another Japanese group (4) revealed that the cytocidal effect of AO-PDT is dependent not only on the wavelength, but also on the illuminance intensity of the xenon light. The illuminance intensity is expressed in units of lux and is measured by a lux meter.

There are many other units to express the luminous intensity or illuminance of a light beam, such as candela (cd), lumen (lm), lux (lm/m^2) and J/cm^2. The units of cd, lm and lux express the illuminance at the illuminated surface at a distance from the light source, while the unit of J/cm^2 expresses the energy intensity of the light source (32). This latter unit (J/cm^2) is the most commonly used for expressing the intensity or energy dose of a laser beam because a laser beam does not lose energy with increasing distance between the light source and the illuminated target. J/cm^2 is correlated with the illuminance at a specific wavelength of 555 nm (32), but not over a wide wavelength spectrum. In AO-PDT, blue light has been shown to yield the strongest cytocidal effect among lights of various colors for the same value of lux, however, unfiltered xenon light yielded an even stronger cytocidal effect than blue light for

the same lux value. Furthermore, this cytocidal effect obtained with the use of unfiltered xenon light increased with increasing value of lux. Unfiltered xenon light has a peak spectrum in the wavelength range of 450 to 550 nm, which is very close to the maximum absorption wavelength of AO fluorescence emission (492 nm). These results suggested that the use of unfiltered xenon light with a stronger illuminance might yield a more portent cytocidal effect in AO-PDT, as compared to even blue light with a weak illuminance.

We demonstrated that a short duration of excitation with blue light, even as short as a minute, was sufficient to kill osteosarcoma cells exposed to AO (33). In the clinical use of AO-PDT using unfiltered light from a xenon lamp (approximately 10,000 lux), we usually use an excitation time of 10 minutes for the surgical area after intra-lesional or marginal tumor resection (1-3). We surmised that if we used a much stronger light source, we might be able to shorten the excitation time to less than 10 minutes. FWL from a xenon lamp was developed for photography in a dark place, and is commonly used to obtain pictures of cars exceeding the speed limit at night. We therefore undertook to investigate whether the cytocidal effect of AO-PDT would be enhanced by the use of FWL in this study. One shot of the FWL used in the study had an illuminance of approximately 1,000,000 lux and a frequency of 60 Hz frequency, whereas CWL has an illuminance of 10,000 lux. Since it would be impossible to compare the luminance intensity of the two lights, we selected the energy intensity, expressed in J/cm^2 which is generally used to compare lights in such study settings (34-36).

The results of the study revealed that the use of FWL, as compared with that of CWL, was associated with a significantly stronger cytocidal effect of AO-PDT on the mouse osteosarcoma cells, for an equal energy intensity as well as excitation time. Therefore, a lower energy intensity and excitation time were required with the use of the FWL, as compared with that of a CWL, for the cytocidal effect of AO-PDT to be observed on the osteosarcoma cells. The results in the control groups confirmed that FWL was not cytotoxic to the osteosarcoma cells in the absence of AO.

Therefore, we concluded that FWL is more useful for AO-PDT than CWL in terms of saving on the excitation time and of obtaining good efficacy of destruction of the residual tumor in the treatment of musculoskeletal sarcomas. In addition, FWL also has the advantage of generating low heat and of having the ability to homogenously illuminate a wider area of more than 225 cm^2 (15 cm in diameter) at a distance of 1 m from the light source; both of these characteristics are favorable for AO-PDT of musculoskeletal sarcomas, because heat destroys normal tissues around the tumor and most sarcomas are larger than 5 cm in diameter.

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