Cytocidal effects of acridine orange evoked by blue light on human bladder cancer cells

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Objectives: Bladder cancer is a major malignancy in urology, and has a high rate of recurrence. Intravesical BCG instillation therapy, despite many adverse effects, is often used to prevent recurrence. Acridine orange (AO), a membrane permeable weakly basic dye, has cytocidal effects when the blue light is illuminated. For this reason, AO was used for the photodynamic therapy (PDT) of musculoskeletal sarcoma. In order to test if AO-PDT can become an effective treatment for bladder cancer, we wanted to determine the cytocidal effects of AO evoked by blue light on the human bladder cancer cell line T-24.

Methods: T-24 cells loaded with AO were observed during blue light illumination under a fluorescent microscope, and vesicle disruption and cell death were assessed by trypan blue staining. To determine the cytocidal mechanism, we determined the change of vesicle disruption and cell death using bafilomycin A1, a vacuolar H+-ATPase inhibitor, and antioxidants, and intracellular distribution of cathepsin D, a lysosomal protease, was also investigated. Finally, the efficacy of the evoked AO on normal rat bladder cells was compared to that on T-24 cells.

Results: Cells showed the disruption of AO-containing vesicles as a green flash during blue light illumination and died within 30 minutes. Bafilomycin A1 and antioxidants inhibited vesicle disruption and cell death. Intravesicular cathepsin D increased in the cytoplasm following illumination. Normal bladder cells showed slight vesicular AO accumulation but did not die in the same condition that almost all T-24 cells died.

Conclusions: AO evoked by blue light has strong cytotoxicity for the T-24 bladder cancer cells and was caused by the lipid peroxidation-mediated disruption of vesicles, where AO is accumulated by vacuolar H+-ATPase, followed by the subsequent release of cathepsin D into the cytoplasm. PDT with AO may be worthwhile for the treatment of bladder cancer.

Key words: bladder cancer cells, photodynamic therapy, acridine orange
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or BCG, 20% to 50% of the patients had recurrence.\textsuperscript{5-10} Moreover, significant side effects were seen in 20% to 30% of the BCG-treated patients, including severe irritative voiding symptoms, gross hematuria, dysuria, granulomatous prostatitis, and urosepsis.\textsuperscript{8,11}

Photodynamic therapy (PDT), using acridine orange (AO) fluorescent dye in particular, is emerging as an optimal therapy for a variety of cancers. AO was extracted from coal tar in the late 19th century as a dye for staining clothes or microorganisms.\textsuperscript{12,13} This material has a unique feature. AO binds densely to acidic vesicles such as lysosomes because AO is membrane-permeable and weakly basic. AO emits red fluorescence in acidic vesicles and green fluorescence in the cytoplasm and nucleus at neutral pH. Accumulation of AO in vesicles causes vesicle disruption when the blue light is illuminated to the cells because the blue light excites AO and then generates reactive oxygen. Due to the vesicle disruption, the cells die.\textsuperscript{14} Clinically, this property has already been used for patients with musculoskeletal sarcomas. Kusuzaki et al. reported that AO-PDT can be used in combination with tumor resection for patients with musculoskeletal sarcoma.\textsuperscript{15} Based on extremely low rates of local recurrence and low rates of limb loss, they concluded that AO-PDT may be a promising approach for the preservation of limb function in musculoskeletal sarcoma cases and may be applicable to many other solid cancers.\textsuperscript{15-17} In spite of these good results in orthopedics, to our knowledge, there was no such report in urology.

Our goal, in the present study, was to determine whether or not PDT with AO is effective and applicable for bladder cancer therapy as an alternative to intravesical BCG instillation. Therefore, we investigated the cytocidal effects of AO evoked by blue light on T-24 bladder cancer cells as an initial biological investigation.

Materials and Methods

Cell culture

We used the T-24 cell line, derived from human bladder cancer, that is widely used in bladder cancer research. The T-24 cell line was purchased from Japanese Cancer Research Resources Bank (Tokyo).\textsuperscript{18} Normal bladder mucosal cells were obtained from adult male C57BL/6 mice (8 weeks old) in accordance with the experimental protocol approved by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine. Mice were euthanized with ether and their bladders were removed. The mucosal layer of the bladder was peeled off and incubated for 15 minutes at 37°C in Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free Hanks' balanced salt solution (Life Technologies, Carlsbad, CA, USA) containing 2.5 mg/ml trypsin (Sigma-Aldrich, St. Louis, MO, USA). Trypsin activity was inhibited by the addition of 0.125 mg/ml trypsin inhibitor (Sigma-Aldrich). After rinsing with enzyme-free Hank's balanced salt solution, the mucosal tissue was triturated using fire-polished pipettes (inner diameter: 0.5 mm) to disperse the cells. Bladder cancer cells and normal bladder mucosal cells were each plated onto poly-L-lysine-coated glass coverslips (Sigma-Aldrich) placed in Petri dishes, left for 5 hours to allow cell attachment, and then cultured in McCoy's 5A medium (American Type Culture Collection, Manassas, VA, USA) containing 10% fetal calf serum in a humidified incubator (5% carbon dioxide, 37°C) for 1 day.

Solutions and Drugs

All biological experiments were performed at room temperature (25°C) in physiological salt solution (PSS), containing 135 mM NaCl, 5 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM HEPES buffered saline, and 5.5 mM glucose. AO (Wako, Osaka) was dissolved in PSS at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 1, and 5 \textmu M. Glutathione, an antioxidant, was dissolved in PSS at 1 mM. Another antioxidant drug \textalpha-tocopherol (vitamin E, Sigma-Aldrich) and the vacular H+-ATPase inhibitor bafilomycin A1 (Wako) were initially dissolved in DMSO (dimethyl sulphoxide) at 100 mM and diluted in PSS at a final concentration of 1 mM and 1 \textmu M, respectively. In all solutions, pH was adjusted to 7.4.

Observation of AO-loaded cells

We investigated the effect of variable concentration, loading time, and washout time of AO on T-24 bladder cancer cells. The T-24 bladder cancer cells cultured on coverslips placed in Petri dishes were pretreated for 30 minutes with PSS. Then, cells were loaded with AO. When the difference of effects induced by different loading time (1, 5, 10, or 15 minutes) was investigated, AO was used at 1 \textmu M. When the concentration-dependency of AO (0.1, 0.2, 0.3, 0.4, 0.5, 1, or 5 \textmu M) was assessed, loading time was fixed at 15 minutes. After loading, the cells were washed with PSS. Then, the coverslip with cells was attached to the underside of a thin chamber (0.5-mm thickness), and another coverslip was attached to the top side of the chamber. The chamber was filled with PSS and placed on the stage of an inverted Zeiss Axiovert 135 TV fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The cells were illuminated with blue light produced by an Osram Mercury Short Arc Photo Optic Lamp HBO 103W/2 passing through an excitation filter (450-490 nm) and a heat-protecting filter.
The light intensity was maintained at 100% of the maximum intensity by an AttoArc device (Carl Zeiss). The light intensity in the illuminated region in the chamber, measured by a portable illuminance meter (LX2, SANWA Electric Instrument, Tokyo), was 150 W/m². The fluorescence was viewed as green through an emission filter (515-565 nm). In order to visualize the red fluorescence of AO accumulating in the vesicles, the fluorescence was excited at 546 nm and emission was monitored at 590 nm. The photomicrographs were taken by a digital camera (AxioCam MRm, Carl Zeiss) driven by AxioVision 4.7 software (Carl Zeiss). After setting each random microscopic field contained 10-25 cells, we observed vesicle disruption in the AO-loaded cells and counted the number of cells with vesicle disruption in each field during a 1-minute blue light illumination. These experiments were repeated 15 times with each AO concentration, AO loading time, blue light illumination time, and AO washout time. Blind counts were performed by three individual observers.

Detection of cell death by trypan blue staining
The cytotoxic effect of AO on bladder cancer cells and normal mucosal cells was determined by trypan blue. These cells were stained with 0.1% trypan blue (Wako) for 1 minute at either 5 or 30 minutes after blue light illumination. The cells stained by trypan blue were regarded as dead cells and counted under microscopy after investigating the vesicle disruption.

Effects of vacuolar H⁺-ATPase inhibitor
The vesicle acidification driven by vacuolar H⁺-ATPase is responsible for filling the vesicles with weakly basic contents. We investigated whether vesicle disruption was suppressed by the pretreatment with the vacuolar H⁺-ATPase inhibitor bafilomycin A1. Cells were pretreated with 1 µM bafilomycin A1 for 30 minutes. After that, the cells were loaded with 1 µM AO for 15 minutes and illuminated with blue light for 1 minute. After setting the random microscopic fields to contain about 10 cells each, we observed vesicle disruption and acute cell death. This experiment was repeated 20 times.

Effects of antioxidant materials
The AO excited by blue light emits fluorescence and generates singlet oxygen (one of the reactive oxygen species), which is produced by energy transfer from the stacked triplet AO to the triplet oxygen, and the singlet oxygen peroxidizes the lipid of the cell membrane. We investigated whether vesicle disruption and acute cell death was suppressed by pretreatment with the antioxidant materials, glutathione and vitamin E. T-24 cells were pretreated with each antioxidant material (glutathione and vitamin E at 1 mM, each) for 30 minutes. Thereafter, the cells were loaded with 1 µM AO for 15 minutes and illuminated with blue light for 1 minute. After setting, the random microscopic fields to contain about 10 cells each, we counted disrupted vesicles and dead cells. This experiment was repeated 20 times.

Immunocytochemistry
As previously explained, acute cell death was caused by destruction of the cell membrane peroxidized with singlet oxygen produced by blue light-evoked AO. We considered another reason for acute cell death could be the destruction of the cell membrane caused by a digestive enzyme released from the disrupted vesicles. We investigated the immunocytochemical localization of cathepsin D, one of the digestive enzymes contained in lysosomes, in AO-loaded cells with or without blue light illumination. The AO-loaded cells with or without blue light illumination were fixed with 4% paraformaldehyde for 20 minutes and washed for 10 minutes with phosphate-buffered saline containing 0.3% Triton-X-100 (PBST), and then treated for 10 minutes with a protein blocking agent (Immunon; Shandon, Pittsburgh, PA, USA) to block nonspecific protein sites. The cells were incubated for 1 hour with rat monoclonal anti-cathepsin D antibody (1:200, R & D Systems, Minneapolis, MN, USA). The antibody was diluted with 0.2% bovine serum albumin, 1% normal goat serum, and 0.1% sodium azide in PBST. After washing with PBS, the cells were incubated for 1 hour with Alexa fluor 488-conjugated goat anti-rat IgG (1:200, Life Technologies). Finally, the cells were washed with PBS. The intracellular localization of AO in the cells and the intracellular immunoreactivity for cathepsin D was investigated in 10 individual fields, set the 10 cells in each site, under a Zeiss LSM510 confocal microscope equipped with argon (488 nm) and helium-neon (543 nm) lasers to excite green and red fluorescence, respectively. A 100X/1.4 oil immersion objective was used. All immunocytochemical procedures were performed at room temperature.

Detection of lipid peroxides
We investigated further using 2-(4-Diphenylphosphanyl-phenyl)-9-(1-hexyl-heptyl)-anthra[2,1,9-def,6,5,10-d'e'f']diisoquinoline-1,3,8,10-tetraone [fluorescent swallow-tailed perylene derivative for detecting lipid hydroperoxides (Spy-LHP)] (Dojindo, Kumamoto) to determine whether or not the blue light-evoked AO generates reactive oxygen and subsequently produces
lipid peroxides, and, if so, whether or not antioxidant materials can prevent the generation of reactive oxygen. The cells were pretreated for 30 minutes with or without bafilomycin A1 or antioxidant materials and then treated with AO (1 μM) for 15 minutes. After blue light illumination for 1 minute, the cells were loaded with Spy-LHP for 1 minute. Green fluorescence in the cells was observed under fluorescence microscope, and the intensity of fluorescence in the cell was evaluated before and after loading with Spy-LHP. The fluorescence intensity was quantified in arbitrary units using NIH ImageJ software. After set the sites of 10 cells approximately, the means of fluorescence intensity in each cell were calculated and the means of fluorescence intensity with or without treatment of bafilomycin A1 and antioxidant materials were compared. This procedure was repeated 10 times per group.

Figure 1. Photomicrographs of T-24 bladder cancer cells loaded with acridine orange (AO). The first panel from the left shows a differential interference contrast (DIC) image of the cells before blue light illumination. The second panel shows the intracellular AO (red), visualized by green light illumination. The third and fourth panels show the changes in intracellular AO (green) during blue light illumination. The time after the start of illumination is indicated at the top of the images. The fifth panel shows a DIC image of the cells stained with trypan blue 30 minutes after blue light illumination. T-24 cells pretreated with (A) PSS, (B) the vacuolar H+-ATPase inhibitor bafilomycin A1, (C) antioxidant vitamin E, and (D) glutathione. Note that AO was not accumulated in acidic vesicles in the (B) bafilomycin A1-pretreated cells. Vesicle disruption was observed in (A) AO-treated cells, but not in (B-D) the cells pretreated with bafilomycin A1, vitamin E, or glutathione. (E) Confocal images of immunocytochemistry with anti-cathepsin D antibody in AO-loaded bladder cancer cells with or without blue light illumination. Green shows cathepsin D immunoreactivity.
Comparison between normal bladder mucosal cells and bladder cancer cells

We investigated the effects of AO on the normal bladder mucosal cells. The concentration and loading time of AO was set to 1.0 μM, and 15 minutes. After setting the site of 10 cells under microscope, we observed vesicle disruption and acute cell death and counted the number of vesicles disrupted and acute dead cells 20 times. The experiments using normal bladder mucosal cells were repeated 10 times after setting the sites of approximately 3 cells.

Analysis and statistics

Percentages of cells with vesicle disruption and dead cells to total cells were calculated. The difference of the mean ± SE of percentage between control and each treatment between bladder cancer cells and normal bladder cells was evaluated by one-way ANOVA (analysis of variance) followed by Bonferroni and Dunn's post hoc test. The difference between mean values with P < 0.01 was considered to be significant.

Results

Vesicle disruption

When the T-24 bladder cancer cells loaded with AO were observed under fluorescence microscopy, AO in the vesicles emitted red fluorescence during green light illumination and AO in the cytoplasm emitted green fluorescence during blue light illumination (Figure 1A). Immediately after the start of blue light illumination, vesicle disruption occurred as a flush of green fluorescence (Figure 1A). This green fluorescence expanded in the cytoplasm and thereafter disappeared gradually. Vesicle disruption was observed in the cells loaded for 15 minutes with AO at more than 0.2 μM and the percentage of the cells showing vesicle disruption increased with increasing concentration (0.2 μM: 19/145 cells, 0.3 μM: 83/155 cells, 0.4 μM: 100/149 cells, 0.5 μM: 225/296 cells, 0.8 μM: 287/299 cells, 1.0 μM: 376/384 cells, 5.0 μM: 203/212 cells. Figure 2A). Almost all the cells showed vesicle disruption at AO concentrations higher than 0.8 μM (Figure 2A). The relationship

Figure 2. Relationships between the percentage of vesicle-disrupted cells and AO concentration, AO loading time, illumination time, and washout time. (A, B) Relationship of the percentage of cells showing vesicle disruption with (A) concentrations of AO and (B) loading time of AO. (C, D) Relationship of the percentage of cells showing vesicle disruption with (C) illumination time and (D) washout time.
between percentages of cells showing vesicle disruption and loading times of AO (1 μM), 1, 5, 10, and 15 minutes, is shown in Figure 2B. After incubations of 5 minutes or longer, almost all the cells showed vesicle disruption (1 min: 59/155 cells, 5 min: 129/139 cells, 10 min: 162/167 cells, 15 min: 150/152 cells). We also investigated the relationship between the percentage of cells with vesicle disruption and blue light illumination time or AO washout time. Vesicle disruption occurred in almost all cells after more than 20-seconds blue light illumination (1 sec: 8/124 cells, 10 sec: 80/120 cells, 20 sec: 116/116 cells, 30 sec: 168/168 cells. Figure 2E). The cells loaded with AO showed vesicle disruption even after 9-hours washout time (1 h: 86/86 cells, 3 h: 72/72 cells, 6 h: 108/126 cells, 9 h: 92/108 cells, 18 h: 10/126 cells. Figure 2G).

Acute cell death

The trypan blue staining at 5 and 30 minutes after blue light illumination showed that the percentage of dead cells increased with increasing concentration at both 5 and 30 minutes (the numbers of dead cells in each concentration AO at 5 minutes and 30 minutes were 0/63, 0/63 cells in 0.1 μM, 20/84, 35/84 cells in 0.5 μM, 26/67, 64/67 cells in 1.0 μM, 26/66, 63/66 cells in 5.0 μM. Figure 2C, D). At all concentrations of AO, the percentage of dead cells at 30 minutes was higher than that at 5 minutes. Investigation of changes in blue light illumination time showed that almost all cells died after 30 seconds or more of illumination with blue light (1 sec: 0/124 cells, 10 sec: 0/120 cells, 20 sec: 12/116 cells, 30 sec: 160/168 cells. Figure 2F). Acute cell death steeply decreased as washout time increased (1 h: 44/86 cells, 3 h: 18/72 cells, 6 h: 10/126 cells, 9 h: 6/108 cells, 18 h: 0/126 cells. Figure 2H).

Effects of vacuolar H+-ATPase inhibitor

No vesicles filled with AO were observed in bafilomycin A1-treated cells (Figure 1B). The mean percentages of dead cells and cells with vesicle disruption assayed at 5 and 30 minutes after illumination in bafilomycin A1-pretreated group was significantly lower than those in the control group (The number of cells with vesicle disruption in the bafilomycin treated cells were 10/231, and no acute cell death was observed in the group of

Figure 3. Relationships between the numbers of acute dead cells and AO concentration, illumination time, and washout time. (A, B) Concentration-dependency of the percentage of dead cells assayed at (A) 5 minutes and at (B) 30 minutes after blue light illumination in AO-loaded bladder cancer cells. (C, D) Relationship of the percentage of dead cells at 30 minutes after blue light illumination with (C) illumination time or (D) washout time.
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Effects of antioxidant materials
In cells pretreated with antioxidant materials, vesicle disruption did not occur and green fluorescence disappeared gradually, though AO in the cytoplasm emitted green fluorescence during blue light illumination (Figure 1C, D). As shown in Figure 3A-C, the percentages of the cells showing vesicle disruption and dead cells assayed at either 5 or 30 minutes following illumination were significantly lower in the glutathione-pretreated group and the vitamin E-pretreated group than those in the control group (In the glutathione-pretreated group, the number of cells with disrupted vesicles were 1/147 cells and acute dead cells were not seen at either 5 or 30 minutes following blue light illumination, and in the vitamin E-pretreated group, were 10/122, 4/122, 8/122.)

Intracellular localization of cathepsin D
In control cells (without blue light), immunoreactivity for cathepsin D was detected in clumps like green fluorescent large grains (Figure 1E, left panel). After illumination with blue light, the immunoreactivity for cathepsin D was observed to infiltrate into the cytoplasm diffusely (Figure 1E, right panel).

Detection of lipid peroxides
The cell images before and after loading with Spy-LHP is shown in Figure 3D. In cells treated with AO alone, after 1-minute blue light illumination, green fluorescence of AO disappeared almost completely, and then when Spy-LHP was added, green fluorescence of Spy-LHP appeared. These phenomena did not occur in the cells pretreated with bafilomycin A1, glutathione or vitamin E. The difference in fluorescence intensity between control cells (non-pretreated cells) and cells pretreated with bafilomycin A1 or antioxidant materials was statistically significant (Figure 3D).

Comparison between normal bladder mucosal cells and bladder cancer cells
As shown in confocal photomicrographs in Figure 4A, normal bladder cells contained a small amount of AO in the vesicles. Vesicle disruption occurred infrequently in normal bladder mucosal cell compared to T-24 cells (The numbers of the vesicle disrupted cells were 1/28 cells, Figure 4B). Furthermore, no acute cell death was seen within 30 minutes after illumination in normal bladder mucosal cells (Figure 4C).
Discussion

The present study is the first investigation aimed at determining if PDT with AO is an effective and applicable for bladder cancer therapy and whether intravesical instillation of AO can take the place of intravesical BCG instillation. The present study clearly showed that the AO evoked by blue light had a cytocidal effect on bladder cancer cells. The vesicle disruption observed as a green fluorescent flush was essentially preceded by cell death.

We further investigated the cytocidal mechanism of the evoked AO on bladder cancer cells. Since AO has a very low molecular weight (M.W. 265) and is a hydrophobic weak base in neutral pH, it rapidly flows into the cytoplasm and lysosomes through the plasma and lysosomal membrane and is trapped by lysosomes, which are acidified by vacuolar H^+-ATPase, due to intraorganella protonation of the AO. We clarified this mechanism by showing the results that the vesicle disruptions were inhibited by the vacuolar H^+-ATPase inhibitor bafilomycin A1 via blocking the intravesicular filling of AO. We also clarified that the blue light-evoked AO produced lipid peroxides by use of Spy-LHP and this phenomenon was inhibited by antioxidant materials. Thus, as previously described,^{14,19,20} the AO is incorporated into acidic lysosomes by the force of vacuolar H^+-ATPase; and the blue light-evoked AO generates reactive oxygen, which causes vesicle disruption.

Vesicle disruption seems to be tightly linked with acute cell death, since the present study indicated that both vesicle disruption and acute cell death showed similar concentration-dependent curves and were inhibited by bafilomycin A1 and antioxidants. Our previous study on malignant melanoma cells also indicates that the cytocidal effect of the evoked AO is caused by vesicle disruption rather than cell membrane blebbing.^{14} Additionally, the release of lysosomal enzyme

![Figure 5.](image-url) Fluorescence images (upper panels) and fluorescence intensity (lower panel) of bladder cancer cells before and after loading Spy-LHP. *P < 0.01, **P < 0.001 vs. control.
into the cytoplasm likely plays a role in cell death. The present immunocytochemistry demonstrated that cathepsin D, a lysosomal protease, infiltrated the cytoplasm after vesicle disruption. Thus, acute cell death was induced by not only direct action of reactive oxygen on lipid membrane but also by the enzyme secondarily released from the disrupted vesicles. The digestive enzyme infiltrating into the cytoplasm could destroy the cell membrane. This was confirmed by trypan blue staining that detected cell membrane-broken cells after illumination.

Finally we examined the cytocidal effect of AO on normal bladder mucosal cells and compared that to the effect on bladder cancer cells. The AO content in normal bladder mucosal cell was slight, and vesicle disruption and acute cell death was not seen in normal bladder mucosal cells. Therefore, the cytocidal effect of AO seems to be more specific to bladder cancer cells.

These results suggested that evoked AO may be useful for the treatment of bladder cancer. Especially in superficial bladder cancer, the efficacy of intravesical instillation of AO is expected to be the same as intravesical BCG treatment without critical adverse effects, such as urinary tract tuberculosis or Reiter's syndrome. AO has already been used clinically in the field of orthopedics, but in order to apply AO to the field of clinical urology further study is warranted.

References


Figure 6. Comparison of bladder cancer cells and normal bladder cells. (A) Confocal images of red fluorescence in AO-loaded cells. (B ,C) The percentage of cells with vesicle disruption and acute cell death. *P < 0.0001 vs. bladder cancer cells.
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