Photodynamic Inactivation of Bladder Cancer Cells (MGH-U1) Sensitized with Acridine Orange and Irradiated by Argon Laser

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ISHIKAWA, S., NEMOTO, R., KANO, S., KOBAYASHI, K. and ISHIZAKA, S. *Photodynamic Inactivation of Bladder Cancer Cells (MGH-U1) Sensitized with Acridine Orange and Irradiated by Argon Laser*. Tohoku J. exp. Med., 1984, 144 (3), 265-271 — Photodynamic inactivation of bladder cancer cells (MGH-U1) was investigated in order to apply laser therapy to the treatment of bladder cancer. After stained with acridine orange (AO), the material cells were irradiated with argon laser. After 24 hr incubation the survival was counted with a hemocytometer. The number of stained cells showed a less than 5% decrease and the number of irradiated (for 15 min) unstained cells showed no decrease compared with untreated cells. Participation of singlet oxygen process in inactivation of MGH-U1 cells was confirmed by the use of D₂O and NaN₃. The result shows that argon laser at the low intensity and with short irradiation time has a sufficient cytocydal effect, suggesting the usefulness of photodynamic inactivation of argon laser with topical use of acridine orange in the treatment of bladder cancer. — photodynamic inactivation; bladder cancer cells; acridine orange; argon laser

It is known that admistration of photosensitizing dyes can result in retardation of tumor growth (Lewis and Goland 1948). Further, Tomson et al. (1974) reported the photodestruction of mouse epithelial tumors after administration of oral acridine orange and argon laser. Recently, Dougherty et al. (1978) reported the photoradiation therapy for the treatment of human malignant tumors by using a hematoporphyrin derivative and red light. Kelly et al. (1975) suggested that photodynamic therapy might be applicable to the treatment of superficial transitional carcinoma of the bladder implanted in mice. On the other hand, Ito and Kobayashi (1974) and Kobayashi and Ito (1976) reported the participation of singlet oxygen molecules in the photodynamic inactivation of yeast cells. In order to apply the argon laser irradiation sensitized with photosensitizing dyes to

clinical use, we have examined the clinical applicability and the participation of singlet oxygen in the photodynamic inactivation, using cultured bladder cancer cells.

MATERIALS AND METHODS

Preparation of cells

The MGH-U1 cell line, established from human bladder cancer (Kato et al. 1977, 1978), was used in the present experiments. Cells were cultured in square culture bottles with DM-160 medium (Kyokuto Co., Tokyo) containing 20% fetal calf serum (Gibco Co., N.Y., USA) at 37°C in a CO₂ incubator.

Sensitization and treatment with D₂O and N₃⁻

Cells were stained with acridine orange (Wako Chemicals, Osaka) for sensitization. In the staining, stock solution of acridine orange (AO) was prepared by dissolving AO in standard saline at a concentration of OD₄₉₂ = 20 (3.5×10^{-4} M). Sample cells (1×10^6 /ml) were stained for 20 min with AO solution at a final concentration of OD₄₉₂ = 1.0 (1.75×10^{-5} M), using the stock solution diluted with phosphate-buffered saline (PBS, NaCl 8 g/liter, Na₂HPO₄ 1.15 g/liter, NaH₂PO₄ 0.2 g/liter, pH 7.40-7.45).

In addition, cells were treated with deuterated medium and azide ion to examine the participation of the singlet oxygen process. In one treatment, the buffer was prepared with deuterium oxide (D₂O, 99.8 atom%, Aldrich Chemical Co., Milwaukee, USA) as a solvent in place of H₂O 15 min prior to laser irradiation. In the other treatment, various amounts of sodium azide (NaN₃, Wako Chemicals, Osaka) were added to give the indicated final concentration 15 min prior to irradiation.

Irradiation with argon laser

Almost all cell populations were irradiated by argon laser (Nippon Electric Co., Tokyo) under the conditions as follows: The wave length of laser was 496.5 nm and the intensity at the sample position was about 4.0 mW/cm². The laser beam was expanded to 3 cm in diameter with a slit and two convex lenses.

Determination of survival

Treated cells with AO and laser were washed four times with PBS and resuspended in medium. All treatments, including sensitization, irradiation and washing were carried out at 16°C in a dark room for about 2 hr. After 24 hr incubation in a CO₂ incubator, cells were removed with a solution of EDTA (0.02%) and trypsin (0.1%), and living cell numbers were counted with a hemocytometer after staining with trypan blue. The experiments were run in triplicate.

Controls

Unirradiated cells were taken as controls for inactivation. They were stained with AO, kept in the same room and washed in the same way. The number of stained cells showed a less than 5% decrease compared with untreated cells.

In the experiment which examined the effect of AO concentration, the cells similarly irradiated but without staining were taken as controls. The number of irradiated (for 15 min) cells without staining showed a less than 1% decrease compared with untreated cells.

RESULTS

Relationship between survival and exposure time

The relationship between survival and exposure time at a fixed concentration

Fig. 1. Relationship between survival and exposure time. MGH-U1 cells, sensitized with AO at a concentration of $1.75 \times 10^{-5} \text{M}$, were irradiated by argon laser. Living cell number was determined 24 hr after irradiation and the percent survival curve was obtained by comparing the living cell number with that of unirradiated cells. Dots are means and the vertical bars indicate the standard deviation.

Fig. 2. Relationship between survival and AO concentration. MGH-U1 cells were irradiated by argon laser for 15 min. The percent survival curve was obtained by comparing the living cell number with that of unstained cells. Dots are means and the vertical bars indicate the standard deviation.

of AO ($OD_{492}=1.0$), is shown in Fig. 1. In this survival curve, 37% survival was at 1.5 min exposure. While there was a small shoulder peak in the curve, the survival decreased almost exponentially.

Relationship between survival and AO concentration

The relationship between survival and AO concentration at 15 min laser exposure is shown in Fig. 2. The decrease in survival curve was remarkable when the OD_{492} of AO was over 10^{-2} , and the concentration of 37% survival was at about $OD_{492}=0.1$.

Enhancement by D_2O

Photodynamic inactivation of cells was enhanced by the replacement of H_2O with D_2O . The result is shown in Fig. 3. At 8 min exposure, the difference in survival under the two conditions was highly significant ($p<0.001$).

Protection by the presence of N_3^- during irradiation

The survival decreased in the presence of NaN_3 during irradiation, as azide ion is known to have cytocydal action as a metabolic inhibitor (Table 1). When NaN_3 was added at a higher concentration, the number of living cell decreased. On the other hand, when NaN_3 was added at a higher concentration to AO staining cells, the number of living cells after irradiation increased. To exclude

Fig. 3. Effects of D_2O on the photosensitized inactivation of MGH-U1 cells. Survival curves ($\circ$ for D_2O and $\bullet$ for H_2O) are shown. Symbols are means and vertical bars indicate the standard deviation.

TABLE 1. *Inhibition effect of azide ion on photodynamic inactivation of cancer cells*

Concentration of NaN_3 (M)	No staining with AO	Staining with AO
	Survival (%)	Survival (%)
0	100	20.8 ± 5.2 (20.8)*
10^{-3}	94.6 ± 9.7	51.8 ± 15 (54.8)*
10^{-2}	82.5 ± 4.6	60.3 ± 7.7 (73.1)*

Irradiation time of laser was 4 min. Values are given as mean $\pm$ s.d.

* Percentage of stained cells over non-stained cells.

the cytocydal effect of NaN_3 as a metabolic inhibitor, percentages of stained cells to non-stained cells were calculated. They were 20.8% without NaN_3 , 54.8% with 10^{-3} M NaN_3 and 73.1% with 10^{-2} M NaN_3 (Table 1). These figures indicated that NaN_3 protected the photodynamic inactivation of MGH-U1 cells. This result confirmed the participation of the singlet oxygen process in photodynamic inactivation.

DISCUSSION

In photodynamic inactivation D_2O was known to lengthen the life time of singlet oxygen (Merkel et al. 1972) and azide ion was known as a specific quencher of singlet oxygen (Nilsson and Kearns 1973). Using the azide ion and deuterated medium, Ito and Kobayashi (1974) and Kobayashi and Ito (1976) proved the participation of singlet oxygen in the photodynamic inactivation of yeast cells sensitized with AO. Furthermore Weishaupt et al. (1976) reported that singlet oxygen was identified as the cytotoxic agent which was probably responsible for in vitro inactivation of TA-3 mouse mammary carcinoma cells following incorporation of hematoporphyrin and exposure to red light. They showed the cytotoxicity of the singlet oxygen using 1, 3-diphenylisobenzofuran as a quencher of photoinactivation of TA-3 cells. In the present experiments, the results of the enhancing effect of D_2O and the protective effect of NaN_3 indicated the participation of singlet oxygen in the photodynamic inactivation of MGH-U1 cells.

In the present study, the dose of argon laser was so low that there was no inactivation of MGH-U1 cells. By staining with AO (1.75×10^{-5} M) followed by argon laser irradiation for 1.5 min, only 37% of the cells survived, showing a remarkable cytocydal effect. The present results show that argon laser at the low intensity and with short irradiation time had a sufficient cytocydal effect suggesting the clinical usefulness of photodynamic inactivation of argon laser with topical use of AO.

A flexible fiberoptic system (Dwyer et al. 1975) can be used to deliver visible laser light to the exposure site, which is a definitive advantage over gamma and

beta rays. In the treatment of bladder tumor, it is advantageous if the urinary bladder is irradiated with a transurethral endoscope.

Systemic use of AO should be avoided due to its toxicity (Van Duuren et al. 1969), but a method of intravesical instillation may be employed in the treatment of bladder tumor. Although transurethral surgery is at present the most effective method in treating superficial bladder tumors, there are a number of problems in treating high stage tumors, recurrent tumors and, in particular, carcinoma in situ (Melamed et al. 1964 ; Barlebo et al. 1972). In views of the pathologic aspect of carcinoma in situ whereby the epithelial cells do not penetrate the basement membrane, we believe photo-inactivation laser therapy promises to be an effective treatment of carcinoma in situ.

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