日レ歯誌 J. Jpn. Soc. Laser Dent. 16：2-9, 2005

Original Article

Experimental Study of Photofrin-mediated Photodynamic Therapy Combined with Sizofiran

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(Accepted for Publication: January 28, 2005)

光化学治療と免疫賦活剤との併用効果に関する研究

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(受理：平成17年1月28日)

要旨：免疫賦活剤のSizofiran (SPG) は，単独投与，放射線化学療法との併用で，腫瘍増殖抑制，延命効果等が報告されている。今回我々は，光化学治療 (PDT) とSPGとの併用効果についてマウス腫瘍を用い検討を行ったので報告する。SPGは10mg/kg/dayを連続10日間，SPGのみおよびSPG + PDT群で腫瘍移植翌日より，PDT + SPG群でPDT翌日から筋肉内投与を開始した。PDTの感光色素にはフォトフリンを使用し，Nd:YAG励起 dye laser照射24時間前に10mg/kgを腹腔内投与した。効果判定には，腫瘍の壊死面積割合，腫瘍組織内のCD45陽性細胞数，TUNEL Labeling Indexおよびマウスの生存率の測定を行った。PDT後にSPGを併用すると，PDTの抗腫瘍効果は増強された。

(J. Jpn. Soc. Laser Dent. 16：2～9, 2005 Reprint request to Dr. NAKAMURA)

Key words = Photodynamic therapy (PDT), Biological response modifier, Photofrin®

キーワード＝光化学治療，免疫賦活剤，フォトフリン®

Introduction

Photodynamic therapy (PDT) is a new modality for cancer treatment[1]. The principle utilized in PDT of cancer dates back to the beginning of the 20th century[2,3]. It involves the activation of a photosensitizing drug that localizes selectively in the tumor by a specific wavelength of light to produce a photochemical reaction in biological systems. The photosensitizer activated under visible light interacts with oxygen which damages organelles, causing cell death.

It is known that both necrosis and apoptosis are responsible for the cell death following PDT[1]. Since Agarwal et al.[4] reported the apoptotic response to PDT, it has been established that PDT can induce apoptosis in vitro and in vivo. Apoptotic induction by PDT depends on cell lines[5,6], the photosensitizer[7,8] and light source, reactive oxygen production[9], the intracellular Ca^{2+} concentration[10], ceramide generation[11], and the dose of each factor[12].

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While it has been known that the basic mode of cell death in PDT is mediated through singlet oxygen generation and other reactive oxygen species, and that direct cellular damage and vascular shutdown contribute to destruction of the tumor, only in recent years has the importance of inflammatory and immune responses been recognized.

The important factor that contributes to the induction of PDT-mediated immune responses is damage generated in cellular membranes and vasculature of the targeted tumor including the adjacent normal tissues. This photooxidative damage results from the extensive release of various potent inflammatory mediators that provoke a prompt and strong inflammatory reaction at the PDT-treated site. The dominant event in such PDT-induced inflammation is rapid and massive invasion of activated inflammatory cells including neutrophils/granulocytes, mast cells, and monocytes/macrophages from the circulation to the PDT-treated site. These cells appear to be the main contributors to the inflammatory-primed immune development process associated with PDT.

Large amounts of cellular debris are generated at a tumor site within a short time following PDT treatment. The particular nature of such material facilitates the uptake and presentation of putative tumor antigens by macrophages and dendritic cells recruited to the tumor site in response to PDT-induced inflammatory signals, ensuring the recognition of tumor-specific epitopes by T lymphocytes and their subsequent activation[1].

Sizofiran (SPG), a biological response modifier used for cancer therapy, has been shown to activate the immune system[13-15]. Leukocytes isolated from mice treated with SPG showed increased response to cytokines and to the mitogen concanavalin A[15]. This drug has been reported to be combined with radiotherapy[16] and chemotherapy[17] for mouse tumors. In these studies, it has been proposed that the potentiation of host defense mechanisms caused by SPG enhances antitumor effects.

The aim of this study was to evaluate the anti-tumor effect of PDT combined with SPG in vivo and to determine an optimal protocol of SPG administration.

Materials and Methods

1. Animals and tumors

Six-week-old, male C3H/HeNCrj mice (Charles River, Osaka, Japan), weighing 20-24g, were used for transplantation of NR-S1 mouse squamous cell carcinomas[18] (By courtesy of National Institute of Radiological Science, Chiba, Japan), that were passaged in the femur. NR-S1 tumor cells in suspension were transplanted subcutaneously at the dorsal region of the mice. The mice were housed in a temperature-controlled room and were fed a chow and water ad libitum. Tumors that grew to 7 × 7 mm or larger in size by 10 days post transplantation were used in all PDT experiment.

2. Photosensitizer

Photofrin (Porphymersodium, Wyeth Lederle Japan, Tokyo, Japan) was dissolved in a sterilized 5 % dextrose solution to a final concentration of 1 mg/ml. The solution was stored at −80℃, and a fresh aliquot was used for each experiment. Photofrin was injected intraperitoneally (10 mg/kg body weight) 24 hrs before laser irradiation.

3. Photodynamic therapy

Red light (wavelength: 630nm) emitted from a pulsed Nd: YAG second harmonic wave pumped dye laser (Quanta-Ray® DCR-3 and PDL-2, Spectra Physics, Mountain View, CA) was used as the light source. A power meter (30 A-P Ophir Optics, Jerusalem, Israel) was used to measure light intensity. In the experimental groups, Photofrin was injected intraperitoneally (10mg/kg body weight), after which the mice were housed in a dark place to avoid light hypersensitivity. Twenty-four hrs later, the tumor was irradiated at a power of 15mJ/pulse/cm^2 for 0, 10, 20, 30, and 40 min (repetition rate, 10Hz; estimated energy dose, 0, 90, 180, 270, and 360J/cm^2). The diameter of the irradiating laser beam entirely covered the tumor. During laser irradiation, animals were under general anesthesia by pentobarbital sodium (50 mg/kg body weight) injected intraperitoneally.

4. Sizofiran treatment

Ten consecutive daily doses of 10 mg/kg of Sizofiran were intramuscularly injected into the hind leg.

5. Experimental protocol

The experiment consisted of five experimental groups (total n = 25), with 5 mice in each group. Group 1 was a control, group 2 (SPG group) was treated by SPG alone, and group 3 (PDT group) was treated by PDT alone. Group 4 (SPG + PDT group) and group 5 (PDT + SPG group) were subjected to combination of PDT and SPG treatment. In group 4, the SPG treatment was initiated 24 hrs after tumor transplantation, and continued for 10 days. In group 5, SPG was administered 24 hrs after PDT, and it was injected daily for 10 days.

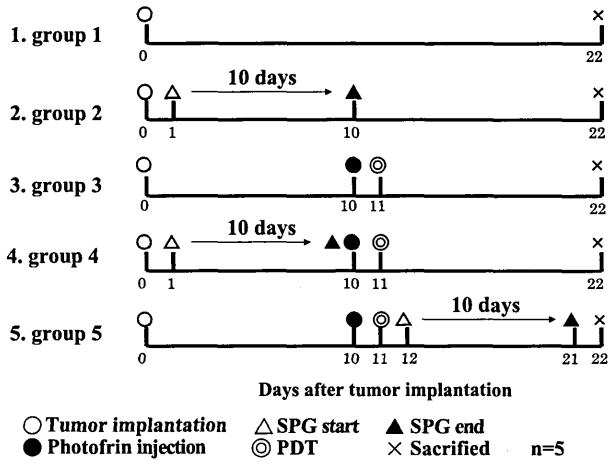

Figure 1　Schema of experimental protocol

The control group did not receive any drugs, or photo-irradiation.

6. Tissue preparation

The animals were euthanized by an overdose of pentobarbital sodium on day 22, and the tumor specimens were harvested and fixed in 3.7% neutral buffered formalin. Processing for routine paraffin embedding was followed by sections being cut to 4 μm thickness and placed on silane-coated glass slides. Five serial sections were used for (1) hematoxylin and eosin (HE) staining, (2) CD45 immunostaining, (3) CD45 immunostaining negative control, (4) TUNEL staining, and (5) TUNEL staining negative control.

7. Terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling

DNA fragmentation was detected by nick-end labeling according to the method of Gavrieli et al.[19] Briefly, the formalin-fixed, paraffin-embedded sections were deparaffinized, washed with phosphate buffered saline (PBS) (pH.7.4), and digested with 20 μg/ml proteinase K (Sigma, St. Louis, MO, USA). Endogenous peroxidase was inactivated using 0.3% H_2O_2 in methanol. Labeling of DNA fragmentations with digoxigenin in the presence of terminal deoxynucleotidyl transferase (TdT), and binding of peroxidase to the reaction site, were performed using the Apop Tag® in situ Apoptosis Detection Kit (Itergen, Norcross, GA, USA). Peroxidase activity was visualized by H_2O_2 and diaminobenzidine (DAB) (Wako Pure Chemicals, Osaka, Japan). The sections were counterstained with methylgreen, dehydrated with ethanol, penetrated with xylene, and enclosed in synthetic resin. For the negative control, sections were treated similarly, but TdT was replaced by PBS.

8. Immunohistochemistry

Immunohistochemical localization of leukocytes was examined using rat anti-mouse CD45 (Leukocyte common antigen, Ly-5) monoclonal antibodies (PharMingen, San Diego, CA, USA) according to the indirect enzyme-labeled antibody method (VECTASTAIN Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA). Deparaffinized and rehydrated sections were immersed in 0.3% H_2O_2 in methanol to block endogenous peroxidase, followed by preincubation with normal rabbit serum in PBS to block nonspecific binding of antibodies. For the negative control, sections were treated similarly, but the primary antibody was replaced by PBS.

Evaluation

1. Percentage area of tumor necrosis

The necrotic area of tumors was measured in the HE-stained specimens on 22 day after tumor implantation using PC-assisted image analysis (Scion Image, Scion, MD, USA). The percentage of necrotic area in relation to the total cross-sectional tumor area was computed[20].

2. Survival time

Survival time (days) of each group was calculated by the Kaplan-Meier method.

3. Growth rate of the tumors

The growth rate of tumors (R) was expressed as R = V1/V2, where V1 is the volume of the tumor at 24 hrs after PDT and V2 is the volume of the tumor just prior to PDT. The volume of the tumor (V) was calculated by modified Gibson et al's method[21], as $V = 4/3\ \pi\ (r/2)^3$, where r is width.

4. TUNEL labeling index

In specimens taken on day 22 for each experimental group and control, the TUNEL labeling index was defined as the percentage of TUNEL-positive cells in 1000 tumor cells counted in four fields selected around the necrotic area. Labeled and unlabeled cells were counted with the aid of a squared eyepiece graticule (Nikon, Tokyo, Japan) at a magnification of 400 ×.

5. Numbers of CD45-positive cells and lymphocytes infiltrating into the tumor tissue

The number of CD45-positive cells infiltrating into the tumor tissue was counted on 22 day after tumor implantation in the specimens. CD45-positive cells in a square field ($0.0625mm^2$) were counted at a magnification of 400× in four selected fields of the tumor tissue. Lym-

phocytes were distinguished morphologically from other CD45-positive cells.

6. Statistical analysis

The data were evaluated by using Fisher's PLSD test for the percentage area of tumor necrosis, growth rate of the tumors, TUNEL labeling index, the number of CD45-positive cells, and the percentage of lymphocytes in the tumor tissue. Survival time was evaluated by the log rank test. The correlation between the TUNEL labeling index of the tumor cells and the number of lymphocytes in the tumor tissue was statistically analyzed by Pearson's correlation coefficient. Results were expressed as mean ± SD (standard deviation).

Results

1. Effects of PDT with varying doses of laser irradiation

Both tumor necrosis and the infiltration of CD45-positive cells were demonstrated to be dependent on the photo-irradiation dose of PDT (Figure 2A, 2B). Photo-irradiation of PDT was shown to affect the growth rate of tumors dose-dependently, reaching a plateau at 270J/cm^2 (Figure 2C). Based on these results, a PDT protocol with 270J/cm^2 irradiation was employed for subsequent experiments.

2. Survival after each protocol

There were no significant differences in survival among the groups (Figure 3).

3. Histological findings and percentage area of tumor necrosis

Necrotic areas were increasingly observed in the experimental groups (Figure 4B) in contrast to the control specimens which showed small necrotic islands (Figure 4A). Group 5 showed the greatest anti-tumor effect, being significantly different from the other groups. Significant differences were also observed between groups 1 and 3, groups 1 and 4, and groups 1 and 5, respectively (Figure 5).

4. Analysis of TUNEL-positive cells

In group 5, many TUNEL-positive cells with condensed nuclei were observed adjacent to the necrotic area (Figure 6A). In group 1, TUNEL-positive cells were occasionally present (Figure 6B). There were significant differences between group 5 and the other groups (Figure 7).

5. Numbers of CD45-positive cells and lymphocytes in the tumor tissue

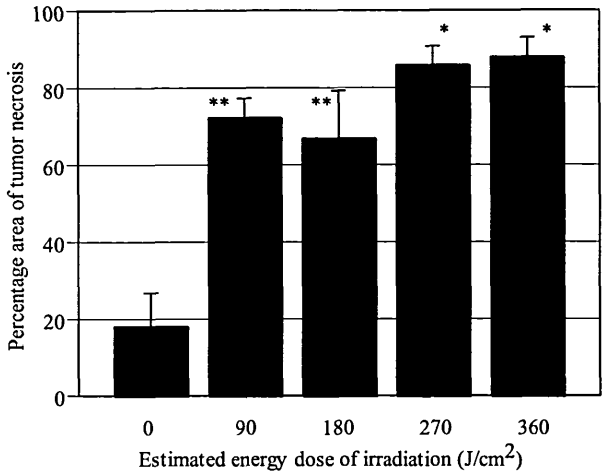

Figure 2A Percentage area of tumor necrosis 24 hrs after PDT. The proportions of the necrotic area in relation to the total cross-sectional tumor area on hematoxylin and eosin-stained sections were calculated. The tumors were treated with various irradiation doses after administration of 10mg/kg of Photofrin®.

* $P < 0.0001$ compared to 0 J. $P < 0.01$ compared to 180J. $P < 0.05$ compared to 90J.

** $P < 0.0001$ compared to 0J. Values are mean ± SD.

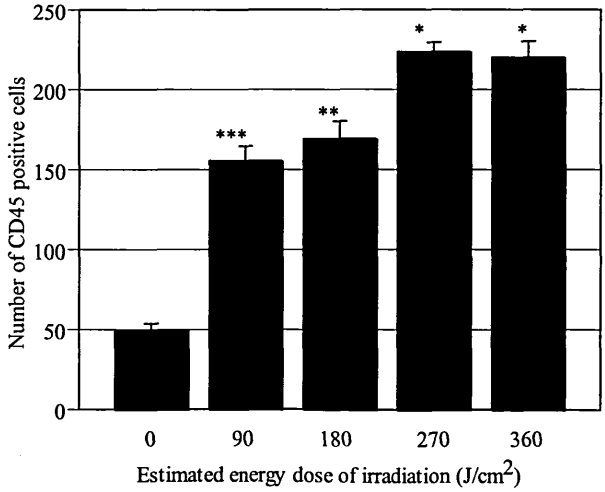

Figure 2B The number of CD45-positive cells in the necrotic area 24 hrs after PDT. CD45-positive cells in a square field (0.0625mm^2) were counted at a magnification of 40 × in four selected fields adjacent to the tumor tissue. The tumors were treated with various irradiation doses in the presence of 10mg/kg of Photofrin®.

* $P < 0.0001$ compared to 180J, 90J, and 0J.

** $P < 0.05$ compared to 90J. $P < 0.0001$ compared to 0J.

*** $P < 0.05$ compared to 0J. Values are mean ± SD.

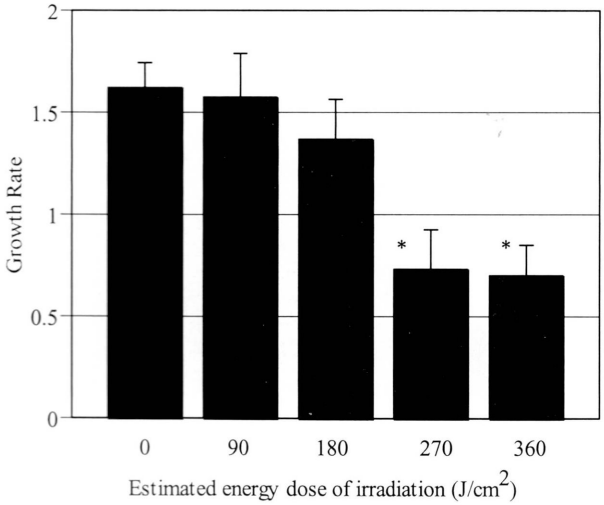

Figure 2C　Effect of PDT on the tumor growth
Growth rate of tumor (R) was expressed as R = V1/V2, where V1 is the volume of the tumor 24 hrs after PDT, and V2 is the volume of the tumor just before PDT. The tumors were treated with varying irradiation doses after administration of 10mg/kg of Photofrin®.
* $P < 0.0001$ compared to 0J and 90J. $P < 0.01$ compared to 180J. Values are mean ± SD.

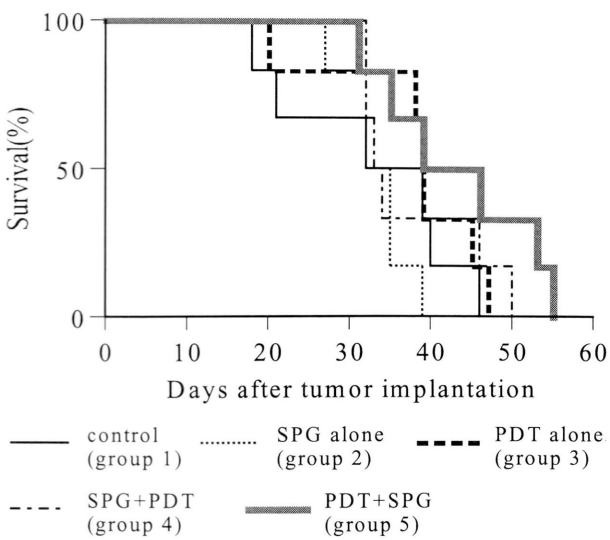

Figure 3　Survival curves
Median survival times were 32.7 ± 11.2, 33.2 ± 3.9, 39.0 ± 9.9, 37.8 ± 8.0, and 43.2 ± 9.7 days in the control (group 1), the SPG alone (group 2), the PDT alone (group 3), the SPG+PDT (group 4), and the PDT+SPG (group 5), respectively.

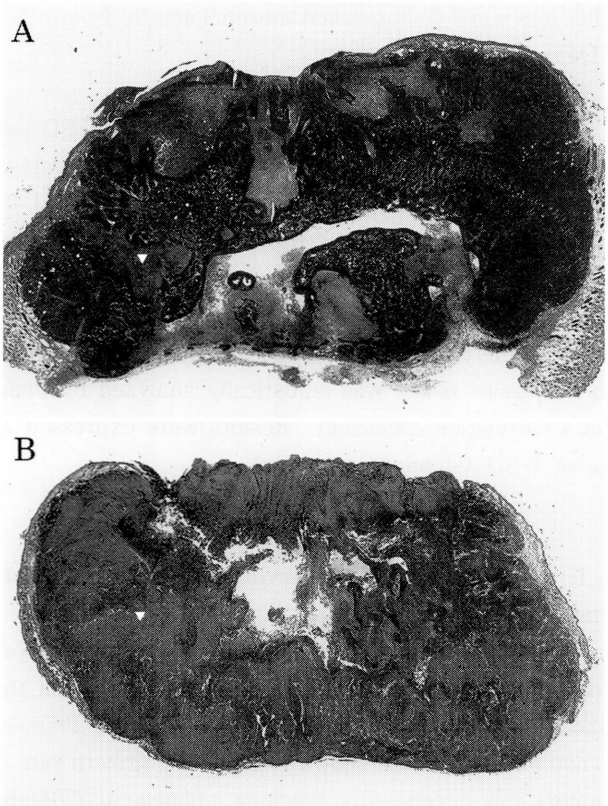

Figure 4　A: Histological findings in the control (group 1) 22 days after tumor implantation. Several necrotic areas (▽) were seen (hematoxylin-eosin stain, original magnification ×4). B: Histological findings 24 hours after the last administration of SPG (22 days after tumor implantation) in a group 5 (PDT+SPG). Necrotic areas (▽) extend from the surface toward the bottom of the tumor. A small area of viable-looking tumor cells is seen only in the peripheral region (hematoxylin-eosin stain, original magnification ×4).

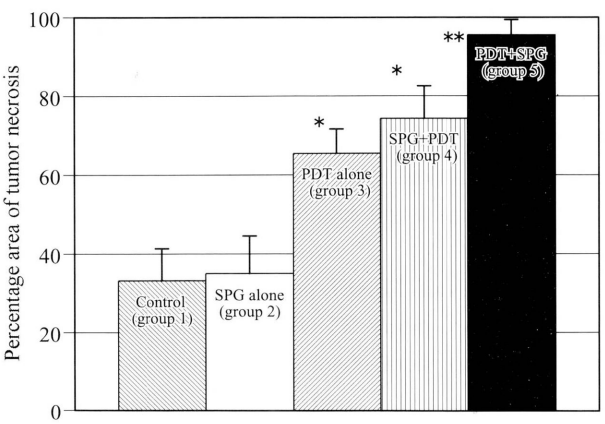

Figure 5　Percentage area of tumor necrosis 24 hrs after the last administration of SPG (22 days after tumor implantation).
* $P < 0.01$ compared to control.
** $P < 0.0001$ compared to control, SPG alone, PDT alone, and SPG+PDT. Values are mean ± SD.

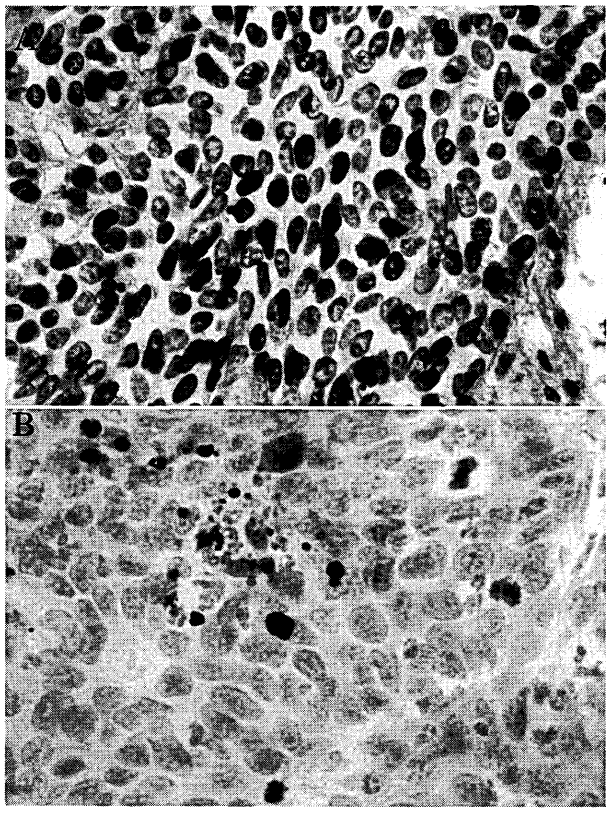

Figure 6 TUNEL staining. Original magnification ×200. A: 24 hrs after the last administration of SPG (22 days after tumor implantation) in group 5 (PDT +SPG). TUNEL-positive tumor cells with condensed nuclei were observed. B: Control (group 1) 22 days after tumor implantation. Only a few TUNEL-positive cells were observed in the tumor tissue.

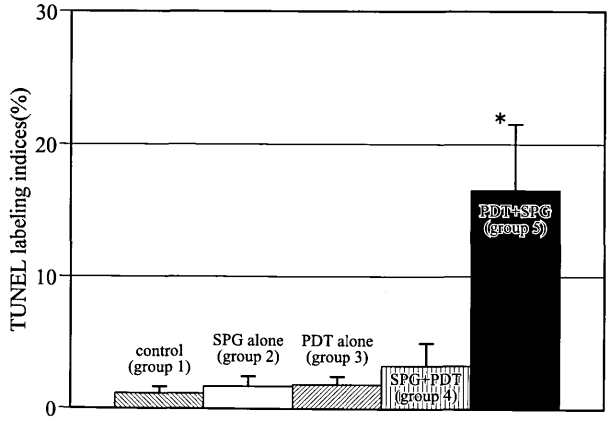

Figure 7 TUNEL labeling index 24 hrs after the last administration of SPG (22 days after tumor implantation). Each labeling index was calculated as the percentage of positive cells in the total cells counted.
* $P < 0.05$ compared to control, SPG alone, PDT alone, and SPG+PDT. Values are mean ± SD.

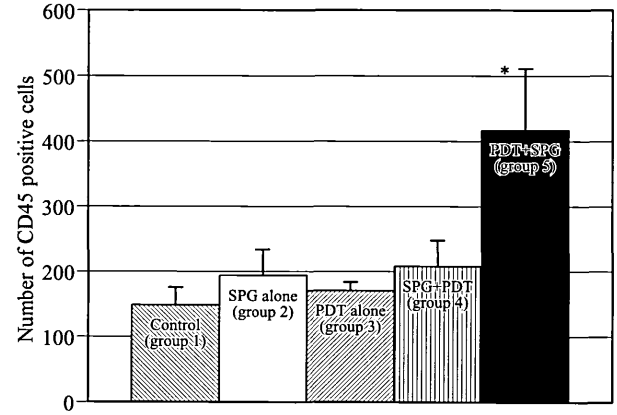

Figure 8 Number of CD45-positive cells 24 hrs after the last administration of SPG (22 days after tumor implantation).
* $P < 0.01$ compared to control, SPG alone, PDT alone, and SPG+PDT. Values are mean ± SD.

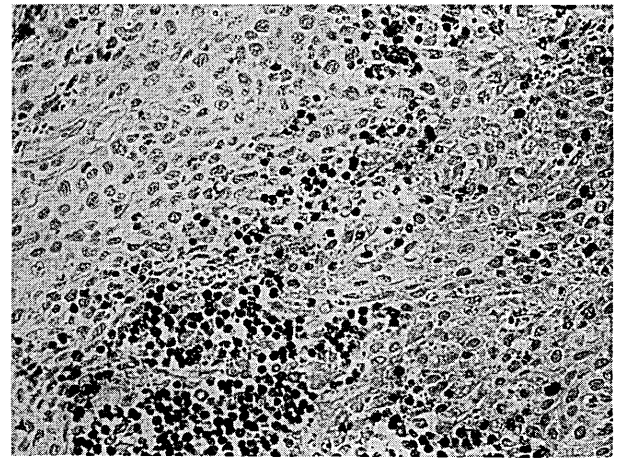

Figure 9 CD45 immunostaining. Original magnification × 200. 24 hrs after the last administration of SPG (22 days after tumor implantation) in group 5 (PDT+SPG). Infiltration of CD45-positive cells into the tumor tissue was observed.

Infiltration of inflammatory cells into the tumor tissue was demonstrated to be predominant in group 5 (Figures 8, 9). The number of lymphocytes was significantly different between group 5 and the other groups (Figure 10).

Pearson's correlation coefficient indicated a significant correlation between TUNEL labeling index and the number of lymphocytes in the tumor tissue ($P < 0.01$).

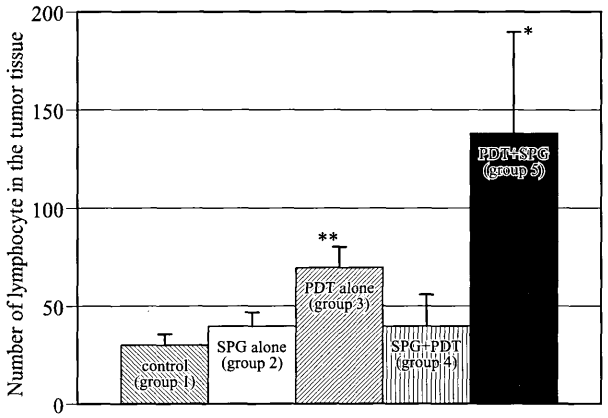

Figure 10　The number of lymphocytes in the tumor tissue 24 hrs after the last administration of SPG (22 days after tumor implantation).
* $P < 0.0001$ compared to control, SPG alone, PDT alone, and SPG + PDT.
** $P < 0.05$ compared to control. Values are mean ± SD.

Discussion

Several studies have reported that the immune response makes an essential contribution to the antitumor effect of PDT[22-25]. Krosl et al. reported that inflammatory infiltration into mouse tumor began within 5 min for neutrophils and within 2 hrs for other types of cells including macrophages after PDT[26].

The inflammatory signaling after PDT may initiate and support the recruitment of leukocytes from the blood and amplify their activity. A massively regulated infiltration of neutrophils, mast cells, and monocyte/macrophages during and after PDT has been documented in studies using tumor models[26,27]. These newly arrived nonspecific immune effector cells will outnumber resident cancer cells. Most notable is a rapid accumulation of a large number of neutrophils. There is increasing evidence that these cells have a profound impact on PDT-mediated destruction of cancerous tissue[31]. Residual neutrophils present in tumor blood vessels overwhelmingly contribute to the damaging process to vascular endothelium. Extravasation of neutrophils also damages the tumor parenchyma. Degranulation of neutrophils results in the release of oxygen-derived free radicals, myeloperoxidase, and lysosomal enzymes. In particular, lysosomal enzymes have a major cytotoxic effect on tumor cells by protease activities[28].

During these processes, neutrophils are also fatally damaged, resulting in the release of chemotactic factors. These chemotactic factors subsequently induce additional infiltration of immune cells. In this study, we recognized a significant increase in both tumor necrosis and the number of CD45-positive cells after PDT (Figure 2A, Figure 2B).

A previous study suggested that the damaged cellular membranes on PDT-treated tumor cells can be recognized by macrophages[29]. After PDT is administered, large amounts of cellular debris generated would be recognized by macrophages as tumor antigen. As shown in Figures 8 and 10, there was a significantly increased number of CD45-positive cells and lymphocytes in the tumor tissue of the PDT + SPG group mice.

The mechanism of SPG immunotherapy differs significantly from cytotoxic chemotherapy. SPG does not exert a direct cytotoxic effect on tumor cells. Its tumoricidal or tumoristatic effect is mediated through indirect stimulation of immune cells such as macrophages, NK cells, and cytotoxic T cells[13-15]. Normally, these cells will attack and lyse tumor cells. PDT, on the other hand, kills the immune cells together with the tumor cells[30]. The different result shown between the SPG + PDT and PDT + SPG groups supports this photodamage explanation.

Previous reports pointed out that tumor cell apoptosis was induced by PDT *in vivo*[31-33]. The interaction between Fas-expressing tumor cells and Fas ligand-expressing lymphocytes has been reported to significantly contribute to the tumor cell killing process[34,35]. In the present study, it is suggested that the predominant response of lymphocytes demonstrated in the PDT + SPG group contributes to the significant tumor cell apoptosis.

The finding of no significant difference in survival time between the control and experimental groups may be due to giving an insufficient dose of PDT, although the PDT + SPG group tended to survive longer compared with the other groups.

In conclusion, the present study revealed that administration of SPG after PDT appears to be a promising approach for improving PDT efficacy in cancer treatment.

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