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Systemic Antitumor Effect of Intratumoral Injection of Dendritic Cells in Combination with Local Photodynamic Therapy

Hisashi Saji,[1,3] Wenru Song,[2] Katsuyoshi Furumoto,[1] Harubumi Kato,[3] and Edgar G. Engleman[1]

Abstract **Purpose:** Photodynamic therapy (PDT), which is used clinically for the palliative treatment of cancer, induces local tumor cell death but has no effect on tumors in untreated sites. The purpose of this study was to determine if local PDT followed by intratumoral injection of naïve dendritic cells (IT-DC) induces systemic antitumor immunity that can inhibit the growth of untreated as well as PDT + IT-DC – treated tumors.

Experimental Design: BALB/c or C57Bl/6 mice were injected s.c. with CT26 colorectal carcinoma cells and B16 melanoma cells, respectively, and following 10 to 12 days of tumor growth, the tumors were treated with PDT alone or PDT followed by IT-DC or IT-PBS. In other studies, tumors were established simultaneously in both lower flanks or in one flank and in the lungs, but only one flank was treated.

Results: Whereas neither PDT nor IT-DC alone was effective, PDT + IT-DC eradicated both CT26 and B16 tumors in a significant proportion of animals and prolonged the survival of mice of which the tumors were not cured. The spleens of mice treated with PDT + IT-DC contained tumor-specific cytotoxic and IFN-γ-secreting T cells whereas the spleens of control groups did not. Moreover, adoptive transfer of splenocytes from successfully treated CT26 tumor-free mice protected naïve animals from a subsequent challenge with CT26, and this was mediated mainly by CD8 T cells. Most importantly, PDT plus IT-DC administered to one tumor site led to tumor regression at distant sites, including multiple lung metastases.

Conclusions: PDT + IT-DC induces potent systemic antitumor immunity in mice and should be evaluated in the treatment of human cancer.

Dendritic cells (DC) are the most potent antigen-presenting cells known, uniquely capable of activating both the cognate and innate arms of the immune system. For example, administration of DCs loaded *ex vivo* with tumor-associated antigens can elicit antitumor immunity resulting in tumor regression in various murine models, and DCs pulsed with tumor derived peptides, proteins, genes or lysates, as well as DCs fused with tumor cells, have all been studied as therapeutic cancer vaccines (1–11). Although the methods are complex and costly to implement, promising results have been obtained in clinical trials in patients with advanced malignancies. These trials have shown DC-based vaccination to be well tolerated and capable of inducing tumor-specific T-cell responses and regression of metastatic disease. On the other hand, the overall therapeutic efficacy of this approach has been limited, indicating a need to either enhance its potency or combine it with other treatment modalities.

Among the modalities that might be combined with DC-based immunotherapy are systemically administered antitumor drugs as well as locally targeted therapies such as radiation, radiofrequency ablation, and photodynamic therapy (PDT). PDT has been approved in the United States and other countries as an anticancer therapy, mainly for the palliative treatment of surgically inaccessible tumors. PDT involves the systemic administration of a photosensitizer that preferentially accumulates in transformed cells, followed by illumination of the tumor with a laser beam (12). In the presence of oxygen, the laser light activates the photosensitizer and initiates a complex photochemical reaction that generates cytotoxic intermediates. Tumor destruction after PDT results from direct cytotoxic effects as well as from the induction of a local inflammatory response (12). Thus, preclinical studies have shown that PDT not only mediates apoptotic and necrotic killing of tumor cells but also alters the tumor microenvironment through the release of proinflammatory cytokines such as tumor necrosis factor α, interleukin (IL)-1 and IL-6 (12, 13).

On the basis of its unique mechanism of tumor destruction, PDT has the potential to create an environment at the tumor site that favors both tumor antigen loading and activation of DCs, key requirements for induction of antitumor immunity (14). Because most tumors lack an abundance of DCs, one way to potentially take advantage of this environment would be to inject a sufficient number of autologous DCs directly into

Authors' Affiliations: Departments of [1]Pathology and [2]Medicine, Stanford University School of Medicine, Palo Alto, California and [3]Department of Surgery, Tokyo Medical University, Tokyo, Japan

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Note: H. Saji and W. Song contributed equally to this work.

Requests for reprints: Edgar G. Engleman, Stanford Blood Center, 3373 Hillview Avenue, Palo Alto, CA 94304-1204. Phone: 650-723-7960; Fax: 650-725-0592; E-mail: edengleman@stanford.edu.

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PDT-treated tumors. Such a strategy alleviates the need to do *in vitro* loading of DCs with tumor antigens because the inflammatory milieu induces DC activation, which facilitates not only antigen acquisition and processing but also migration of the DCs to draining lymph nodes where they interact with a broad range of potential effector cells. In the current study, we evaluated the effect of combining PDT with intratumoral injection of syngeneic DCs (IT-DC) on two histologically distinct murine tumors, CT26 colon carcinoma and B16 melanoma. The results show that this combined treatment induces strong and durable tumor-specific immunity that results in the destruction not only of targeted tumors but also of tumors at distant sites.

Materials and Methods

Mice. Female BALB/c (H-2^d) and C57Bl/6 (H-2^b) mice, 6 to 8 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the Stanford animal facility in accordance with the NIH guidelines.

Cell lines. The murine CT26 colon carcinoma, B16 melanoma (F10 clone), MAD109 lung carcinoma, and EL-4 lymphoma cell lines used in this study were maintained in complete RPMI 1640 with 10% fetal bovine serum, penicillin G (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (10 mmol/L).

DCs. Bone marrow–derived DCs were generated in the presence of granulocyte macrophage colony-stimulating factor and IL-4 for 7 days as previously described (15). Bone marrow cells were harvested from femurs and tibias, and after RBC lysis, the resulting cell suspension was incubated in complete RPMI 1640 containing recombinant murine granulocyte macrophage colony-stimulating factor (10 ng/mL; PeproTech, Inc., Rocky Hill, NJ) and recombinant murine IL-4 (10 ng/mL; PeproTech). On day 2, nonadherent granulocytes were gently removed and fresh medium with granulocyte macrophage colony-stimulating factor and IL-4 was added. On day 5, loosely adherent cells were dislodged and replated. On day 7 of culture, the unpulsed DCs were collected. The maturational status and percentage of DCs were verified by flow cytometry, and staining of three surface markers (CD11c, CD86, and class II antigen) showed the purity of DCs to be ≥74%.

Photosensitizer and laser unit. ATX-S10 Na(II), a hydrophilic chlorin photosensitizer with an absorption maximum at 670 nm (16), was obtained from Photochemical Co. Ltd. (Okayama, Japan). A diode laser (Hamamatsu Photonics, Hamamatsu, Japan) was used as a light source for exciting ATX-S10 Na(II). The diode laser is a continuous-wave laser operating at 670-nm wavelength.

During the light irradiation, mice were anesthetized with ketamine (125 mg/kg: Vedco, Inc., St. Joseph, MO) and xylazine (25 mg/kg; Phoenix Pharmaceutical, Inc., St. Joseph, MO) and were restrained in a specially designed holder.

Combined PDT and IT-DC therapy of CT26 colon cancer. Preliminary studies with ATX-S10 Na(II) and a diode laser, were carried out on the basis of a published protocol (16) to identify a drug dose and laser setting for inhibition of growth of CT26 tumors *in vivo* without major local or systemic toxicity. CT26 tumor cells (10^6 per mouse) in 100 μL HBSS were injected into the lower right flank of BALB/c mice. On day 12, when the average tumor volume was 153.0 ± 11.5 mm^3, the mice received an i.v. injection of ATX-S10 Na(II) (5 mg/kg body weight), followed 4 to 5 hours later by 150 J/cm^2 laser irradiation of the tumor. DCs (1×10^6 per injection in 50 μL PBS) were injected into the tumor on days 13, 14, 15, and 17. The tumor volume was measured thrice a week using a caliper [tumor volume (mm^3) = (longer diameter) × (shorter diameter)^2 × 0.4]. Animals were sacrificed when the tumor diameter exceeded 20 mm or when there were signs of animal distress. Survival was recorded as the percentage of surviving animals on a given day. Surviving mice had no sign of tumor when experiments were terminated.

Combined PDT and IT-DC therapy of B16 melanoma. For the B16 melanoma tumor model, C57Bl/6 mice were inoculated s.c. with 5×10^5 tumor cells in the lower right flank. On day 10, mice with established tumors (average tumor volume, 80.0 ± 5.4 mm^3) were treated with 150 J/cm^2 laser irradiation to the tumor 4 to 5 hours after an ATX-S10 Na(II) (5 mg/kg body weight) injection and given intratumoral injections of DCs on days 11, 12, 13, and 15 (1×10^6 per injection in 50 μL PBS). The measurement of tumor volume and survival was as above.

ELISPOT assays. ELISPOT assays were done with an ELISpot mouse IFN-γ system (R&D Systems, Inc., Minneapolis, MN) according to the instructions of the manufacturer. Splenocytes were isolated 4 weeks after tumor inoculation. After lysis of RBC, splenocytes were resuspended at a final concentration of 5×10^5/mL and 100 μL of this suspension were then incubated at 37°C for 24 hours in ELISPOT plates coated with anti-IFN-γ with 100 μL medium with or without irradiated (30 Gy) stimulator cells (CT26, MAD109, B16, or EL-4).

Cytotoxicity assays. Cytotoxicity was measured by a standard chromium-51(^{51}Cr) release assay. Splenocytes were harvested 4 weeks after tumor inoculation. After lysis of RBC, splenocytes (1×10^6/mL) were stimulated *in vitro* by irradiated (100 Gy) tumor cells (1×10^5/mL) at 37°C for 5 days in the presence of 10 units/mL IL-2. Following culture, splenocytes were separated from dead cells and debris with Lympholyte-M cell separation media (Cederlane Laboratories, Inc., Hornby, Ontario, Canada). Target cells were labeled with ^{51}Cr (200 μCi/5×10^6 cells) for 1 hour at 37°C, washed, and then incubated in U-bottomed wells with effector cells at various effector-to-target cell ratios at 37°C for 4 hours. Spontaneous release and maximum release were determined by incubating target cells in medium alone or in 1% SDS, respectively. Spontaneous release was always <20% of maximum. Radioactivity was counted in a liquid scintillation counter and the percentage of specific target cell lysis was calculated with the formula $[(E - S) / (T - S)] \times 100$, where E is the average experimental release, S is the average spontaneous release, and T is the average maximal release.

Adoptive transfer of splenocytes. To determine whether lymphocytes induced by PDT + IT-DC could protect naïve animals from a tumor challenge, BALB/c mice were inoculated s.c. with CT26 cells and treated with PDT + IT-DC as before. Four weeks later, the splenocytes were harvested and 1×10^7 cells were infused i.v. into naïve mice. Control groups of mice received splenocytes from CT26 tumor-bearing mice treated with either IT-PBS alone, IT-DC alone, or PDT + IT-PBS. One day later, these mice were s.c. challenged with a lethal number (1×10^6) of CT26 cells and monitored for tumor volume and survival.

To analyze the role of specific T-cell subsets in tumor protection, splenocytes were harvested from inoculated tumor-free mice treated with PDT + IT-DC, and $CD4^+$ or $CD8^+$ T cells were depleted by using CD4 (L3T4) or CD8a (Ly-2) coupled microbeads and magnetic cell sorting (Miltenyi Biotec, Inc., Auburn, CA). Splenocytes, $CD4^+$ T-cell–depleted splenocytes, or $CD8^+$ T-cell–depleted splenocytes (1×10^7) were infused i.v. into groups of five naïve mice. Control mice received splenocytes from naïve mice. One day later, the mice were s.c. challenged with a lethal number (1×10^6) of CT26 cells and monitored for tumor volume and survival. These depletion conditions were validated by flow cytometry analysis using anti-CD4 (L3T4)-FITC and CD8a (Ly-2)-phycoerythrin monoclonal antibodies (PharMingen, San Diego, CA). The percent depletion of $CD4^+$ and $CD8^+$ cells was 97% and 93%, respectively.

Secondary tumor challenge. To determine the persistence of tumor-specific immunity in the mice treated by PDT + IT-DC, at day 60 after first tumor inoculation, mice showing complete regression of CT26 tumors were given a second s.c. tumor challenge (1×10^6 CT26) in the left lower flank (contralateral to the first injection site). These mice, as well as a control group of naïve mice that were inoculated with 1×10^6 CT26 tumor cells, were monitored for tumor size and survival.

Effect of PDT + IT-DC on contralateral tumors. To determine whether PDT + IT-DC treatment of one s.c. tumor affected an

established contralateral s.c. tumor, CT26 cells (1×10^6) were injected s.c. into both lower flanks of BALB/c mice on day 0. On day 12, tumor-bearing mice were either untreated or treated with combined PDT + IT-DC (using the protocol above) into the tumor on the right side but not into the tumor on the left side. The bilateral tumor-bearing mice were followed for tumor volume on both flanks.

Effect of PDT + IT-DC on multiple distant tumors. BALB/c mice were inoculated s.c. with 1×10^6 CT26 tumor cells on day 0. On day 5, these mice were injected i.v. with 1×10^6 CT26 tumor cells. PDT + IT-DC was administered on day 12 (as above) to the s.c. tumor alone. On day 21 after i.v. tumor inoculation, mice were euthanized and lungs were harvested and fixed with Bouin's solution (Sigma-Aldrich, St. Louis, MO).

Statistical analysis. Differences between groups were analyzed using unpaired two-tailed Student's *t* test. Survival curves were plotted by the Kaplan-Meier method and comparisons among groups in the survival data were calculated by log-rank test.

Results

Effect of combination treatment with PDT and IT-DC on CT26 tumors. To test the hypothesis that local PDT followed by IT-DC can inhibit primary tumor growth, BALB/c mice were injected s.c. with CT26 tumor cells. On day 12, the tumors reached an average diameter of 10.5 mm and could not be cured with PDT alone (data not shown). PDT was done at that time and syngeneic DCs were injected intratumorally 1 day later (day 13) and again on days 14, 15, and 17. Although the tumors grew rapidly following treatment with PBS alone, IT-DC alone, and PDT + IT-PBS, the combination of PDT + IT-DC resulted in significant suppression of tumor growth ($P < 0.05$ versus all other groups; Fig. 1A). Four of the nine (44%) animals treated with PDT + IT-DC became tumor-free and the overall survival of the PDT + IT-DC group was significantly prolonged compared with other groups (log-rank test, $P = 0.0006$; Fig. 1B). To determine if the combination of PDT + IT-DC results in immunologic memory, mice that had been treated with PDT + IT-DC and were tumor-free following an initial tumor inoculation were rechallenged in the opposite flank with a second lethal inoculation of the same tumor. The results show that these mice not only survived but were completely resistant to this second inoculation (Fig. 1C).

Effect of combination treatment with PDT and IT-DC on B16 tumors. In contrast to CT26 tumors, the B16 melanoma is considered poorly immunogenic and highly aggressive. Moreover, PDT for the treatment of melanoma has had only limited benefit (17), which is attributed to the presence in such tumors of a large amount of light-absorbing melanin pigment that prevents penetration of the laser beam into the tumor tissue. Our preliminary studies showed that PDT alone could not induce any suppression of growth of B16 tumors, even against s.c. tumors as small as 3 mm in diameter (data not shown). Surprisingly, PDT in combination with IT-DC resulted in a striking antitumor effect ($P < 0.05$, versus all other groups; Fig. 2A). Four of seven (57%) mice treated with PDT + IT-DC became tumor-free and the overall survival of the PDT + IT-DC group was significantly prolonged compared with other groups (log-rank test, $P = 0.0004$; Fig. 2B). Interestingly, two of four PDT + IT-DC–treated tumor-free mice developed white hair at sites of treatment, and in one of these mice, white hair could be seen at a site (neck) distant from the PDT site at ~40 days after treatment (Fig. 2C).

In vitro *characterization of the antitumor immune response induced by PDT + IT-DC.* A correlation between *in vitro* tumor-specific IFN-γ production by host-derived T cells and systemic antitumor immunity has been shown in other studies (18, 19). Using ELISPOT assays, we evaluated whether treatment of CT26 tumor-bearing mice with PDT + IT-DC could elicit tumor-specific IFN-γ-secreting T cells. As shown in Fig. 3A, splenocytes from mice treated with PDT + IT-DC contained significantly more tumor-specific IFN-γ-secreting cells than splenocytes from other groups ($P < 0.05$, versus other groups). Moreover, this cytokine was not secreted spontaneously or in response to

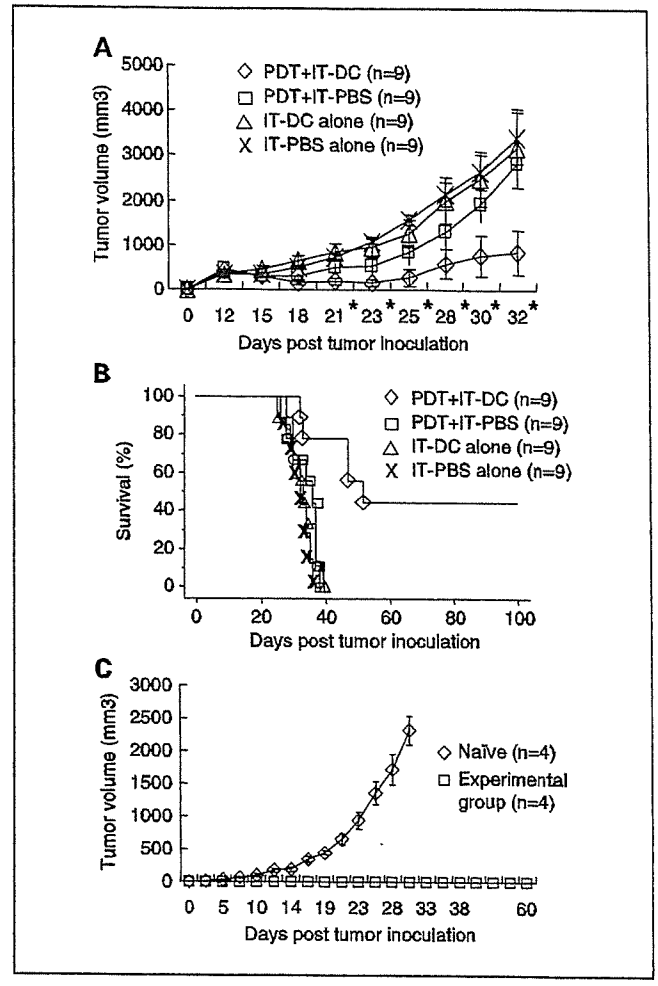

Fig. 1. Effect of combined PDT + IT-DC on the growth of established CT26 syngeneic colon carcinoma tumors. CT26 cells (1×10^6) were injected s.c. in the right lower flank of BALB/c mice. On day 12, mice with established tumors (mean tumor volume 153.0 ± 11.5 mm^3) were treated with PDT as described in Materials and Methods. DCs (1×10^6 in 50 μL PBS) were administered intratumorally on days 13, 14, 15, and 17. The experimental groups included intratumoral injection of PBS alone (50 μL, $n = 9$; ×); intratumoral injection of DCs alone ($n = 9$; △); PDT combined with intratumoral PBS ($n = 9$; □); and PDT combined with IT-DC ($n = 9$; ◇). *A,* mean tumor volume (mm^3) for treatment groups [mean tumor volume = (longer diameter) × (shorter diameter)^2 × 0.4]. Points, mean; bars, SE. *, $P < 0.05$, PDT + IT-DC versus other treatments. *B,* survival of mice recorded as the percentage of surviving animals on a given day. Surviving mice had no sign of tumor when the experiment was terminated. *C,* CT26 tumor rechallenge of tumor-free mice after PDT + IT-DC. Mice that had received CT26 inoculation followed by PDT + IT-DC were rechallenged s.c. with a lethal number (1×10^6) of CT26 tumor cells ($n = 4$; □). Naïve mice inoculated s.c. with the same number of CT26 cells served as controls ($n = 4$; ◇). Experiments were done thrice with similar results.

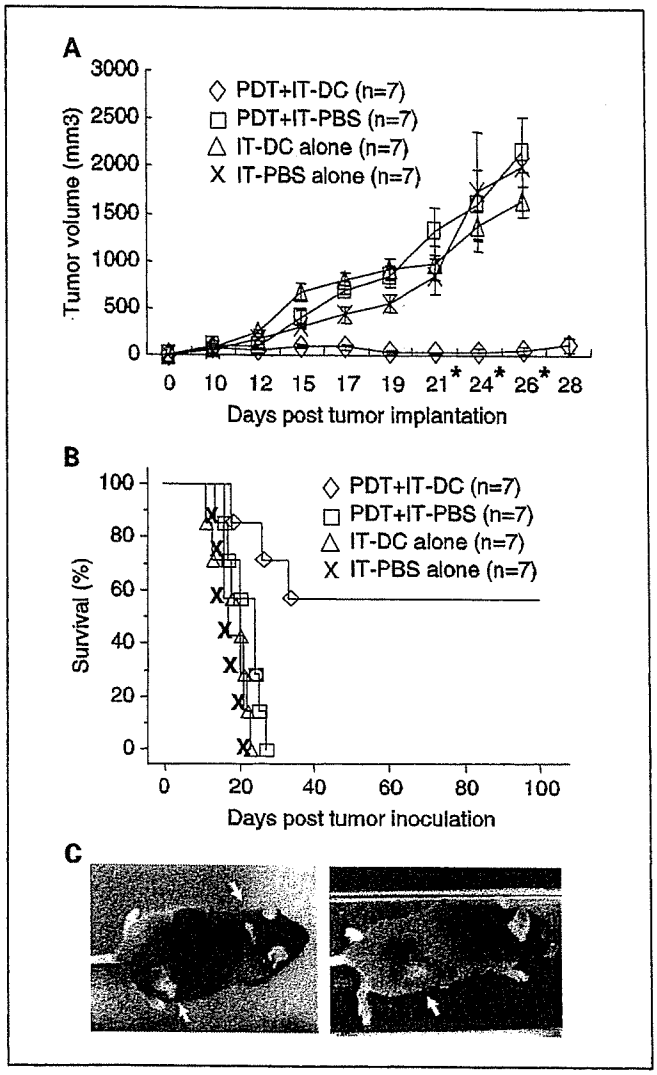

Fig. 2. Effect of PDT + IT-DC on the growth of established B16 melanoma tumors. B16 cells (5×10^5) were injected s.c. in the right lower flank of C57Bl/6 mice. On day 10, mice with established tumors (average tumor size, 80.0 ± 5.4 mm^3) were treated with PDTand received IT-DC (1×10^6 in 50 μL PBS) on days 11, 12, 13, and 15. The experimental groups included IT-PBS alone (50 μL, $n = 7$; ×); IT-DC alone ($n = 7$; △); PDT + IT-PBS ($n = 7$; □); and PDT + IT-DC ($n = 7$; ◇). *A*, mean tumor volume (mm^3) for treatment groups [mean tumor volume = (longer diameter) × (shorter diameter)^2 × 0.4]. Points, mean; bars, SE. *, $P < 0.05$, PDT + IT-DC versus other treatments. *B*, survival of mice recorded as the percentage of surviving animals on a given day. Surviving mice had no sign of tumor when the experiment was terminated. Experiments were done thrice with similar results. *C*, photographs of tumor-free mice taken 60 days after PDT + IT-DC treatment of B16 melanoma. Arrows, white hair growing in an untreated site (neck of mouse on left) and in treated sites (right flanks of both mice).

MAD109 cells, which are irrelevant syngeneic murine lung tumor cells, indicating that the observed response was immunologically specific to CT26 tumor cells. In additional studies, we analyzed the splenocytes of the different treatment groups for the presence of CT26-specific CTLs. Figure 3B shows that such cells were present in the PDT + IT-DC group but not in other groups ($P < 0.05$, versus all other groups). There was no killing of a syngeneic lung cancer cell line (MAD109), indicating that the CTLs are CT26 tumor specific (data not shown).

A tumor-specific immune response was also observed in B16 tumor-bearing mice that had been treated with PDT + IT-DC. As shown in Fig. 4A, splenocytes from such mice contained significantly more tumor-specific IFN-γ-secreting cells compared with splenocytes from control groups ($P < 0.05$). In addition, as shown in Fig. 4B, PDT + IT-DC induced tumor-specific CTLs, as indicated by the presence of such cells in treated but not control animals. No lysis of a syngeneic lymphoma cell line (EL-4) was observed, indicating those CTLs are B16 tumor specific (data not shown).

In vivo ***characterization of the antitumor immune response induced by PDT + IT-DC.*** To further evaluate the role of CTLs in PDT + IT-DC–induced tumor protection, splenocytes from CT26 inoculated PDT + IT-DC–treated tumor-free mice and splenocytes from control groups were transferred to naïve mice. One day later, the mice were inoculated with a lethal dose of

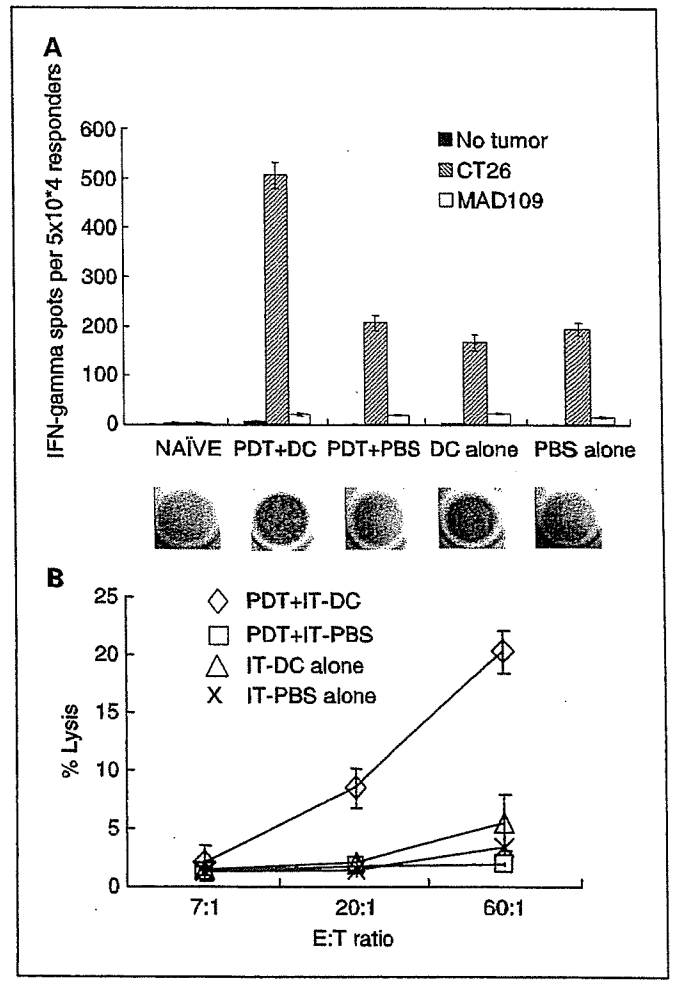

Fig. 3. Induction of *in vitro* anti-CT26 tumor immunity by PDT + IT-DC. *A*, CT26 tumor-bearing mice were treated as detailed in the legend to Fig. 1. Four weeks after tumor inoculation, splenocytes from treated, control, and naïve mice were incubated with or without specific tumor cells or MAD109, irrelevant irradiated tumor cells in an IFN-γ ELISPOTassay. Columns, average number of spots per 5×10^4 responders of triplicate samples; bars, SE. *, $P < 0.05$, versus other groups. Representative ELISPOT wells are shown below the graph. Data are from one of three representative experiments. *B*, CTLs in mice that had been inoculated with CT26 tumor cells followed by treatment with PDT + IT-DC. Four weeks after tumor inoculation, graded numbers of splenocytes from mice receiving various treatment protocols (×, IT-PBS alone; △, IT-DC alone; □, PDT + IT-PBS; ◇, PDT + IT-DC) were cultured in the presence of irradiated CT26 cells for 5 days. Cytotoxicity was measured with a standard 4-hour ^{51}Cr release assay at various ratios of effectors to targets using ^{51}Cr-labeled CT26 cells as targets.

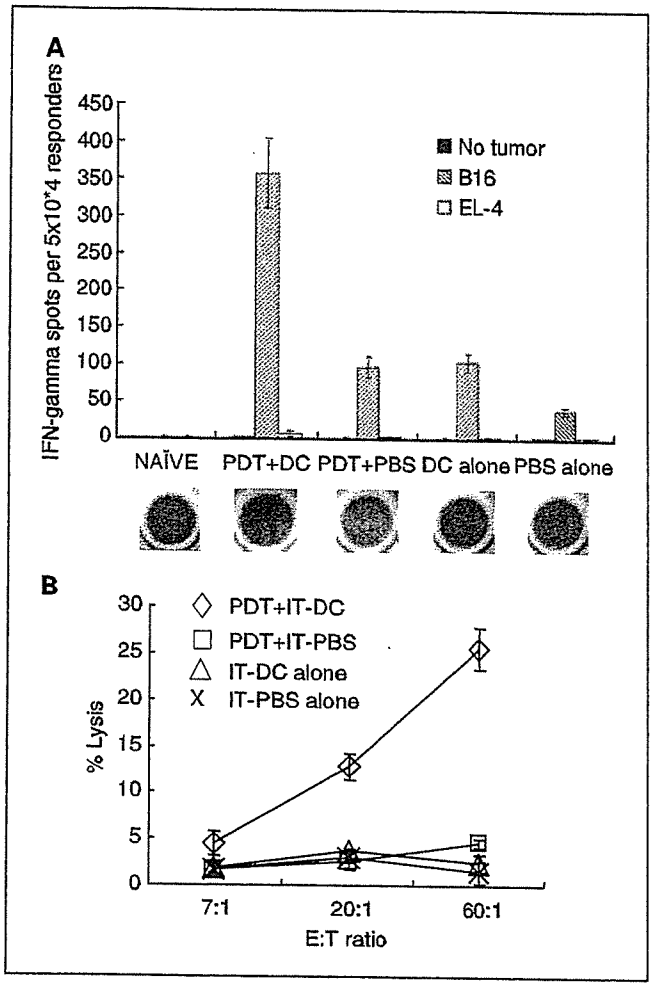

Fig. 4. Induction of anti-B16 tumor immunity by PDT + IT-DC treatment. *A*, B16 tumor–bearing mice were treated as detailed in the legend to Fig. 2. Four weeks after tumor inoculation, splenocytes from treated, control, and naïve mice were incubated with or without specific target cells or irrelevant irradiated tumor cells (EL-4) in an IFN-γ ELISPOT assay. Columns, average number of spots per 5×10^4 responders of triplicate samples; bars, SE. *, $P < 0.05$, versus other groups. Representative wells from an ELISPOT plate are shown below the graph. Data are from one of three representative experiments. *B*, CTLs in mice that received B16 tumor inoculation followed by treatment with PDT + IT-DC. Four weeks after tumor inoculation, graded numbers of splenocytes were cultured in the presence of irradiated B16 tumor cells for 5 days (×, IT-PBS alone; △, IT-DC alone; □, PDT + IT-PBS; ◇, PDT + IT-DC). Cytotoxicity was measured with a 4-hour ^{51}Cr release assay at various ratios of effectors to targets using ^{51}Cr-labeled B16 cells as targets.

CT26 tumor cells. Mice receiving splenocytes from the PDT + IT-DC–treated mice, but not from other mice, were protected from a subsequent tumor challenge with CT26 (Fig. 5A). To determine the role of $CD4^+$ and $CD8^+$ T cells in tumor protection, we repeated this experiment using whole splenocytes or splenocytes depleted of $CD4^+$ or $CD8^+$ T cells. Splenocytes from naïve mice were used as negative controls. One day later, these mice were s.c. challenged with a lethal number of CT26 tumor cells. As shown in Fig. 5B, whereas both $CD4^+$ and $CD8^+$ T cells from PDT + IT-DC–treated mice contributed to tumor protection, CD8 T cells mediated most of the effect.

Effect of PDT + IT-DC on distant untreated tumors. To determine whether treatment of one tumor with PDT + IT-DC conferred systemic antitumor effects, CT26 tumors were established simultaneously in both lower flanks, but only one site was treated with PDT + IT-DC. As shown in Fig. 6A, the growth of both tumors was significantly suppressed compared with the control group, showing that treatment of a primary tumor with PDT + IT-DC confers suppression of the growth of untreated as well as treated tumors. To simulate the clinical scenario in which multiple tumors are present at sites distant from the primary tumor, mice were simultaneously inoculated s.c. in one flank (as above) and i.v. with CT26 tumor cells. This resulted in the seeding of both lungs and the growth of multiple pulmonary metastases. Although a few lesions were visible in the lungs of mice treated with PDT + IT-DC, as shown in Fig. 6B, PDT + IT-DC treatment of the s.c. tumor in such mice resulted in a significant reduction of the lung lesions compared with untreated control animals ($P < 0.05$). Representative examples of lungs from a healthy mouse, a tumor-bearing PDT + IT-DC–treated mouse, and an untreated (control) tumor-bearing mouse are shown in Fig. 6C, D, and E.

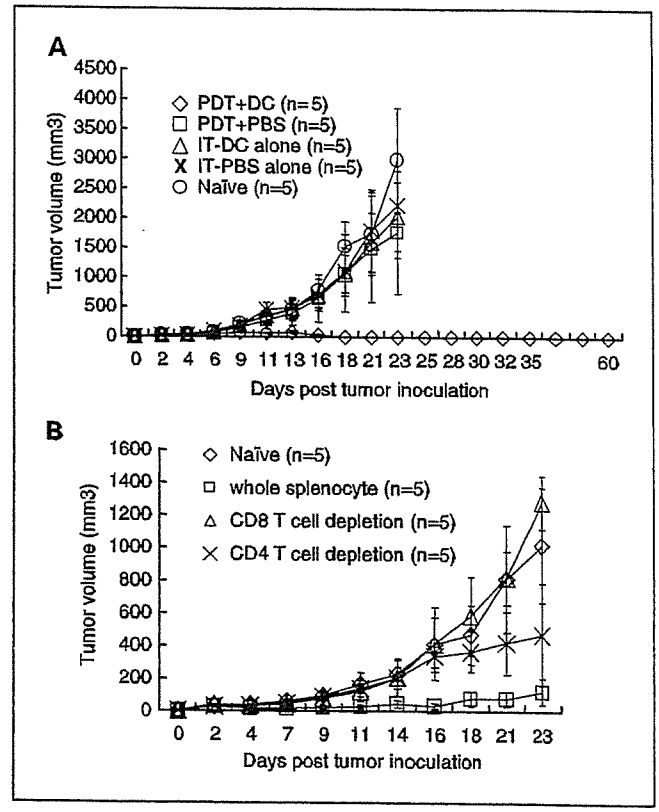

Fig. 5. *A*, adoptive transfer of splenocytes from PDT + IT-DC–treated mice to naïve mice prevents CT26 tumor growth. Splenocytes (1×10^7) from CT26 inoculated mice treated with IT-PBS alone (×), IT-DC alone (△), PDT + IT-PBS (□), or PDT + IT-DC (◇) were infused i.v. into naïve mice. One day later, the mice were challenged s.c. with a lethal number (1×10^6) of CT26 tumor cells. Naïve mice without splenocyte transfer were used as a control (○). Points, average tumor volume (mm^3) of five mice per group; bars, SE. *B*, role of CD4 and CD8 T-cell subsets in protection against CT26 tumors. Splenocytes were harvested from tumor-free mice treated with PDT + IT-DC. $CD4^+$ or $CD8^+$ T cells in splenocytes were depleted by using CD4 (L3T4) or CD8a (Ly-2) microbead magnetic cell sorting. After magnetic selection, whole splenocytes (□), CD4+ T-cell–depleted splenocytes (×), and CD8+ T-cell–depleted splenocytes (△) were infused i.v. into each of five naïve mice. Mice treated with splenocytes from naïve mice served as a control group (◇). One day later, these mice were s.c. challenged with a lethal number (1×10^6) of CT26 cells and monitored for tumor volume and survival.

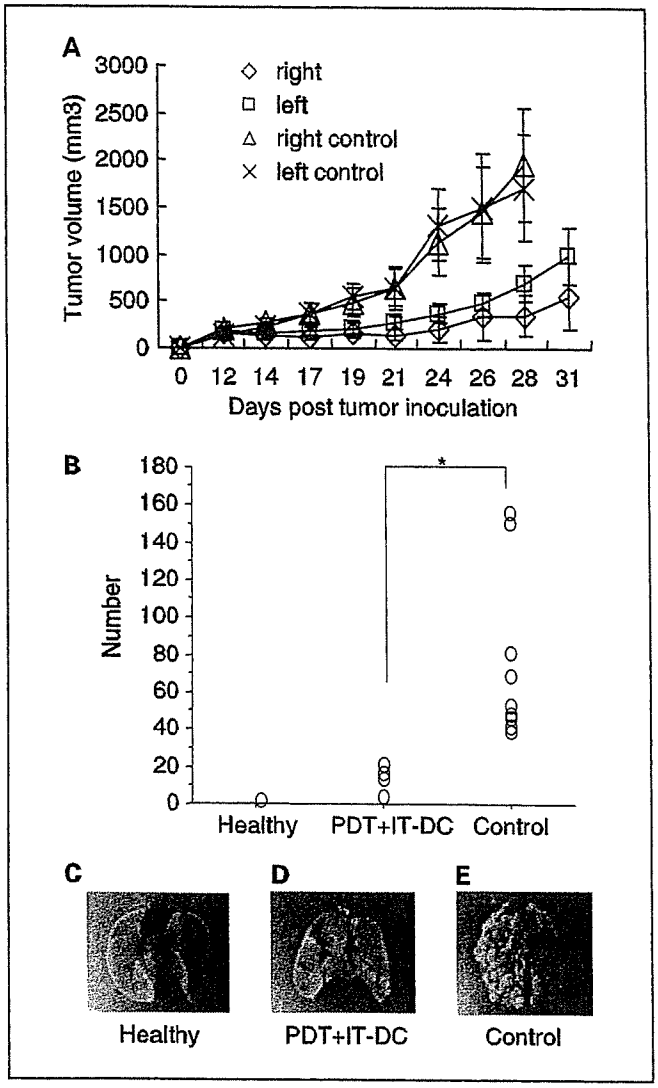

Fig. 6. Systemic tumor-specific immunity induced by local PDT + IT-DC treatment affects tumors at distant sites. *A*, CT26 tumor cells (1×10^6 for each side) were s.c. inoculated in both lower flanks of BALB/c mice. Twelve days later, the tumor-bearing mice were either untreated or treated with PDT + IT-DC into tumors on the right side but not the left side. Tumor growth on the left (untreated) and right (treated) sides was monitored and average tumor volume (±SE) was determined. Shown is the tumor volume on the right (with IT-PBS; △) and left (×) sides of control mice and the right (◇) and left (□) sides of treated mice. Each group contained five mice. *, $P < 0.05$, between left treated and control groups and between right treated and control groups. *B*, 10 BALB/c mice were inoculated s.c. with 1×10^6 CT26 tumor cells on day 0. On day 5, mice were injected i.v. with the same number of CT26 tumor cells, and on day 12, the s.c. tumors were treated with PDT + IT-DC. On day 26, lungs were harvested and stained with Bouin's solution to confirm and quantify lung metastases. *, $P < 0.05$, versus control group. *C*, representative lungs from a healthy mouse. *D*, representative lungs from a tumor-bearing PDT + IT-DC – treated mouse. *E*, representative lungs from an untreated control mouse.

Collectively, these data indicate that PDT + IT-DC therapy induces potent systemic tumor-specific immunity against CT26 colon cancer.

Discussion

One reason postulated for the limited clinical efficacy of most DC-based cancer vaccines studied to date is their variable ability to induce strong antitumor immunity, particularly CTL responses. This variability may have been due to problems related to tumor antigen selection or DC activation. Most DC-based clinical trials have included only a single or few tumor antigens although tumors contain thousands of potential antigens. Moreover, although a wide range of methods have been used to activate DCs and load them with antigens *in vitro*, there is no agreement about which of these methods induces optimal antitumor immunity. We sought to overcome these limitations by introducing unpulsed syngeneic DCs directly into tumors following treatment of the tumors with PDT, which creates a microenvironment that favors tumor antigen acquisition as well as activation of the DCs. The results confirm that PDT-treated tumors contain all of the factors necessary to activate DCs, load them with antigens, and induce an effective systemic antitumor immune response.

Although relatively little work has been done to evaluate the combination of PDT and IT-DC, several previous studies have shown the benefit of combining IT-DC with chemotherapy or radiotherapy (20–26). In contrast to chemotherapy or radiotherapy, PDT is not associated with systemic toxicity. Moreover, PDT renders murine tumors more immunogenic than tumors treated with UV or ionizing irradiation, or frozen and thawed tumors (27). Recently, Jalili et al. (28) reported that PDT in combination with IT-DC had little or no effect on s.c. CT26 tumors but inhibited the growth of contralateral tumors. By contrast, we observed dramatic effects on both local and distant tumors despite the fact that treatment was begun 12 days after inoculation of mice with a higher tumor dose than that studied by Jalili et al. One possible explanation for this surprising result is that we injected the same DC number four times as opposed to twice in their study. Another difference in our two studies is that Jalili et al. used the hematoprophyrin derivative, profimersodium (Photofrin), as a photosensitizer whereas we used ATX-S10 Na(II). Although Photofrin is widely used clinically, its potency is limited by weak absorbance at the shorter range of the red region of the spectrum. In addition, Photofrin is not a pure substance but a mixture of hematoporphyrin monomers, dimers, oligomers, and their dehydration products, and these products are associated with long-lasting skin photosensitivity (12). In contrast, ATX-S10 Na(II) is a homogeneous agent that preferentially accumulates in tumor tissues and is eliminated from normal tissues within 24 to 48 hours after injection. Moreover, its absorption maximum lies at 670 nm, which is longer than that of Photofrin (630 nm) and enables deeper tissue penetration (16). Using ATX-S10 Na(II) as a photosensitizer, we showed that PDT in combination with IT-DC inhibits the growth of two histologically distinct murine tumors. Interestingly, treatment of B16 tumors with combined PDT + IT-DC was at least as effective as it was for CT26, despite the poor immunogenicity of B16 and its well-documented resistance to PDT alone (29).

Our studies clearly show that PDT + IT-DC induces systemic antitumor immunity as well as tumor-specific immunologic memory. In the B16 model, the observation of white hair in untreated sites of mice, of which the tumors had been eradicated following PDT + IT-DC, suggests that treatment induced a systemic immune response against one or more shared antigens present in normal melanocytes as well as B16 tumors. In the CT26 model, PDT + IT-DC treatment of a single s.c. tumor resulted in regression of both contralateral as well as multiple

pulmonary tumors. The cells responsible for mediating tumor regression were cytotoxic T cells as indicated by both *in vitro* cytotoxicty assays and the observation that naïve animals were protected against tumors by adoptively transferred splenocytes from successfully treated tumor-bearing mice. Depletion of specific T-cell subsets (CD4 or CD8) in the adoptively transferred splenocytes indicated the CD8 T cells are the major effector cells induced by the PDT + IT-DC treatment.

Critical to the systemic antitumor effect of PDT + IT-DC is the capture of tumor-associated antigens by DCs as well as DC activation. Whether necrotic or apoptotic tumor cells serve as the superior source of tumor-associated antigens is controversial (30–34). Our data (not shown) strongly suggest that PDT, which induces both apoptosis and necrosis of tumors (12, 28), causes DCs to take up and process tumor antigen released by the dying tumor cells, mature, and become activated *in situ* and then cross-prime T cells against tumor-derived antigens. Interestingly, PDT alone had little or no effect on the growth of B16 tumors and analysis of such tumors following their treatment with PDT revealed a much smaller percentage of apoptotic and necrotic cells than in identically treated CT26 tumors (data not shown). Because PDT + IT-DC was effective as a treatment for both tumors, it seems likely that a relatively small number of dead or dying tumor cells can provide the necessary antigens required for DC-mediated induction of antitumor immunity. Furthermore, because it is known that PDT stimulates the expression of inflammatory cytokines such as tumor necrosis factor α, IL-1, and IL-6 (12, 13), perhaps the presence of such factors in the tumor microenvironment played a critical role in the induction of DC maturation.

In summary, the data presented in this report indicate that PDT + IT-DC results in potent systemic antitumor immunity and regression of tumors including tumors at sites distant from the treated site. Based on these findings, this novel regimen may prove beneficial in the treatment of patients with advanced metastatic disease as well as in the neoadjuvant setting before resection of tumors known to have a high recurrence rate.

Acknowledgments

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Immunohistochemical Differential Diagnosis Between Large Cell Neuroendocrine Carcinoma and Small Cell Carcinoma by Tissue Microarray Analysis With a Large Antibody Panel

Jun-ichi Nitadori, MD,[1,2,4] Genichiro Ishii, MD,[1] Koji Tsuta, MD,[1] Tomoyuki Yokose, MD,[1] Yukinori Murata, MT,[1] Tetsuro Kodama, MD,[3] Kanji Nagai, MD,[2] Harubumi Kato, MD,[4] and Atsushi Ochiai, MD[1]

Key Words: Lung cancer; Large cell neuroendocrine carcinoma; Small cell carcinoma; Tissue microarray; Immunohistochemistry; Large antibody panel

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Abstract

To elucidate additional phenotypic differences between large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC), we performed tissue microarray (TMA) analysis of surgically resected LCNEC and SCLC specimens. Immunostaining with 48 antibodies was scored based on staining intensity and the percentage of cells that stained positively. Four proteins were identified as significantly expressed in LCNEC as compared with SCLC: cytokeratin (CK)7, 113 vs 49 ($P < .0301$); CK18, 171 vs 60 ($P < .0008$); E-cadherin, 77 vs 9 ($P < .0073$); and β-catenin, 191 vs 120 ($P < .0286$). Immunostaining of cross-sections containing LCNEC and SCLC components revealed significant expression of CK7, CK18, and β-catenin in the LCNEC component compared with the SCLC component in 2 of 3 cases. Our results indicate that significant expression of CK7, CK18, E-cadherin, and β-catenin is more characteristic of LCNEC than of SCLC, and these findings provide further support that these tumor types are separate entities morphologically and immunophenotypically, if not biologically.

Lung cancer is a major cancer throughout the world and the most common cause of cancer mortality. The revised World Health Organization (WHO) classification of lung cancer published in 1999 classifies neuroendocrine tumors into 4 major histologic categories: low-grade malignant "typical" carcinoid, intermediate-grade malignant "atypical" carcinoid, and 2 high-grade tumors, large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC).[1] In 1991, Travis et al[2] introduced the term *large cell neuroendocrine carcinoma* to describe a distinct category of high-grade neuroendocrine tumor with biologic and light microscopic characteristics different from those of high-grade SCLC. Morphologically, LCNEC is characterized by neuroendocrine morphologic features (rosette formation), large tumor cells (3 times larger in diameter than a small resting lymphocyte) with a low nuclear/cytoplasmic ratio, numerous nucleoli, a high mitotic rate (>10 in 10 high-power fields), a large degree of necrosis, and immunohistochemically staining positive for one or more neuroendocrine markers.

Some authors have reported that LCNEC has a poorer prognosis than SCLC,[3,4] whereas others have reported finding no significant difference in outcome between LCNEC and SCLC.[5-7] SCLC is sensitive to chemotherapy, but the optimal therapy for LCNEC has yet to be defined. Demetri et al[8] advocated that LCNEC be treated in a manner similar to SCLC but acknowledged that there may be a greater role for surgical resection in LCNEC. Nevertheless, it remains unclear how patients with LCNEC should be treated. Until now, few investigators have attempted to identify differences in molecular expression between LCNEC and SCLC. Sturm et al[9] reported a significantly higher frequency of thyroid transcription factor (TTF)-1 positivity with SCLCs, but no other biologic markers