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Photodynamic therapy with recombinant adenovirus AdmIL-12 enhances anti-tumour therapy efficacy in human papillomavirus 16 (E6/E7) infected tumour model

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Summary

Immunotherapy with photodynamic therapy (PDT) offers great promise as a new alternative for cancer treatment; however, its use remains experimental. Here we investigated the utility of adenoviral delivery of interleukin-12 (AdmIL-12) as an adjuvant for PDT in mouse tumour challenge model. PDT was performed by irradiating Radachlorin in C57BL/6 mice transplanted with TC-1 cells. PDT plus AdmIL-12 treatment for tumour suppression as well as specific immune responses were evaluated with the following tests: *in vitro* and *in vivo* tumour growth inhibition, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) assay, and cytotoxic T lymphocyte (CTL) assay. Direct intratumoral injection of AdmIL-12 resulted in a significant suppression of tumour growth compared to the control group. Treatment of PDT along with AdmIL-12 further enhanced antitumour effects significantly higher than either AdmIL-12 or PDT alone. This combined treatment resulted in complete regression of 9-mm sized tumour in every animal. We also evaluated immune responses induced by these treatments. Combined treatment significantly increased the production level of IFN-γ and TNF-α compared with that by AdmIL-12 or PDT alone. PDT plus AdmIL-12 enhanced antitumour immunity through increased expansion of the CTL subset mediated by $CD8^+$ T cells. Taken together, these results indicate that the high anti-cancer activity of PDT with AdmIL-12 is a powerful tool against cancer therapy and is a promising subject for further investigation.

Keywords: human papillomavirus; IFN-γ; photodynamic therapy; Radachlorin; recombinant adenovirus AdmIL-12

Introduction

Photodynamic therapy (PDT) is a method of treating malignant tumours based on the principle of photodynamic damage to tumour cells through a photochemical reaction.[1] Several chemicals have been used as photosensitizers of PDT for a wide range of malignant tumours as well as non-malignant diseases. The locality of photodynamic damage to a tumour is caused by the photosensitizer's ability to accumulate in tumour tissue as a result of the directed, localized, and precise laser irradiation.[2] PDT clinical trials with a variety of photosensitizers demonstrate great promises in treating malignancies of the oesophagus, bronchus, brain, peritoneal cavity, skin, bladder, and head and neck; as well as in treating non-oncological disorders, such as age-related macular degeneration.[3,4] Although the clinical results of PDT are usually positive, this therapeutic modality has not been well optimized yet and tumour recurrences often occur after treatment.[3–5]

Chlorins have represented the second generation of photosensitizers with promising physicochemical properties and high photodynamic efficiency.[6] Radachlorin is especially known to be effective when excited by optical radiation at a wavelength of 662 ± 5 nm.[7] As a result, it starts to actively generate cytotoxic elements (such as singlet oxygen) in the tumour. Its main active component purpurin 5 is hydrophobic. It was combined with hydrophilic chlorine, enhancing chemical stability in an aqueous solution. All chlorin derivatives except

Radachlorin were less effective than m-tetrahydroxyphenylchlorin, the well-known photosensitizer.[8] Pharmacokinetics and biodistribution studies have shown that maximal tumour accumulation of Radachlorin was achieved at 5 and 0·5 hr after intraperitoneal (40 mg/kg) and intravenous (20 mg/kg) administration, respectively.[9] The full clearance period has been found to be 48 hr after intraperitoneal administration after any route of administration, a very important point as regards the problem of skin phototoxicity.[10] Therefore, Radachlorin shows excellent characteristics to be used in PDT.

Interleukin-12 (IL-12), a T helper 1 (Th1) cytokine, is well-known to induce a cellular immune response and suppress tumour growth.[11] Immunoregulation by IL-12 is of central importance in cell-mediated immune responses against pathogens and tumours that are controlled by cell-mediated mechanisms.[11,12] Recently, it has been demonstrated that inhibitors of cyclooxygenase (COX)-2, hypoxia-inducible factor-1 alpha, and vascular endothelial growth factor (VEGF) can be effective in combination with PDT, and IL-12 plays a major role in the suppression of these genes.[13,14]

In this study, we firstly showed the enhanced antitumour effect of PDT using Radachlorin with recombinant adenovirus AdmIL-12 using mice bearing tumours caused by human papillomavirus (HPV) 16 E6/E7 oncogene expressed TC-1 tumour cells. The present study showed that the combination therapy of PDT plus AdmIL-12 was much more effective on the suppression of tumour growth, compared with PDT or AdmIL-12 alone and it was mediated by the cytotoxic T lymphocyte (CTL) response and increased production of interferon-γ (IFN-γ) from $CD8^+$ T cells.

Materials and methods

Photosensitizer and PDT

Radachlorin was purchased from the RADA-PHARMA group (RADA-PHARMA Co, Ltd., Moscow, Russia). PDT was carried out as previously described.[15] Briefly, the light source was a diode laser with a 662 ± 3 nm wavelength (Won-PDT D662; Won Technology, Daejeon, Korea). Female 4–6-week-old C57BL/6 mice were purchased from Daehan Biolink (Daejon, Korea) and were maintained in a specific pathogen free (SPF) animal facility at The Catholic University of Korea. TC-1 cells (2×10^5) were injected subcutaneously into the right flank of C57BL/6 mice. After TC-1 tumour cells had made a tumour size of 9 mm in mean diameter, the mice were given intratumoral injection with 1×10^8 plaque-forming units (PFU) of adenoviruses expressing LacZ or IL12 in a final volume of 100 μl. After 2 days, tumour-bearing mice were intravenously injected with Radachlorin (10 mg/kg). Three hr after injection, the animals were anaesthetized and the tumours were irradiated with 300 J/cm^2 with a light fiber inserted into the tumour mass. Tumour growth was measured twice a week using calipers, and recorded as volume diameter [longest surface length (a), width (b) and height (c), $(a/2) \times (b/2) \times c \times 3{\cdot}14$].

Cell cultures

TC-1 cells derived from primary epithelial cells of C57BL/6 mice cotransformed with HPV-16 E6 and E7 as well as c-Ha-ras oncogenes were cultured and maintained as previously described.[16]

Preparation of recombinant AdLacZ and AdmIL-12

Recombinant adenoviral vector containing an IL-12 gene (AdmIL-12) and LacZ gene (AdLacZ) under the control of the cytomegalovirus promoter was kindly provided from Dr Y. C. Sung (POSTECH, Pohang, Korea). Adenovirus was propagated and prepared as previously described.[16]

Cell morphology

TC-1 cells were plated onto eight-well chamber slides in a volume of 100 μl (5×10^6cells/well). Twenty-four hr later, Radachlorin was added at a volume of 100 μl/well with the concentrations of 0, 1·25, 2·5, and 5 μg/ml. After a predetermined time, the Radachlorin solution was discarded and cells were washed three times with saline, and then media were added at a volume of 100 μl/well. The cultures were then subjected to laser irradiation (6·25 J/cm^2). Morphological changes were examined under a JEOL 100/CX electron microscope.

Measurement of Radachlorin uptake

Tumour-bearing mice were killed at 0·5–24 hr after intravenous injection of a 10 mg/kg dose of Radachlorin. Tumours were harvested and weighed before the measurement of the Radachlorin concentration. For the quantification of Radachlorin concentrations, the minced tumour samples were homogenized in phosphate-buffered saline (PBS) with a tissue homogenizer. Cell debris was removed by centrifugation (13 680 ***g***) and fluorescence (recorded at 642 nm after excitation at 595 nm) was measured with Multilabel Counter 1420 (Perkin-Elmer, Downers Grove, IL).

CTL assay

Splenocytes were stimulated for 5 days in the presence of 20 units/ml of interleukin 2 (R&D Systems, Wiesbaden Norderstedt, Germany) with TC-1 cells, treated

previously for 3 hr with mitomycin C (30 μg/ml). CTL assays were performed in a standard 5 hr ^{51}Cr release assay.[16]

Enzyme-linked immunosorbent assay (ELISA)

One millilitre aliquot containing 5×10^6 splenocytes was added to wells of 12-well plates. Then, 1×10^5 TC-1 cells treated previously with mitomycin C (30 μg/ml for 3 hr) were added to each well. 3 days later, cell supernatants were secured and then used for detecting levels of IFN-γ and tumour necrosis factor-α (TNF-α) using commercial cytokine kits (R&D Systems).

In vivo *depletion of CD4$^+$ and CD8$^+$ T cells*

For *in vivo* cell depletion, anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) ascites fluids were generated by injecting hybridoma cells (American Type Culture Collection, Manassas, VA) into pristane-primed nude mice i.p. One hundred μl of ascites fluids were administered i.p. on days −3, 0, and 3 of tumour challenge. Antibody treatment resulted in >98% depletion of specific CD4$^+$ and CD8$^+$ T-cell subsets of representative animals over a 3-week period. Depleted mice were subsequently challenged with tumour cells on day 0.

Statistical analysis

Statistical analysis was performed with ANOVA and Student's *t*-test. Values between the different groups were compared. *P* values of less than 0·05 were considered significant.

Results

Radachlorin uptake

To see the accumulation level of Radachlorin in tumours, we measured the concentration of Radachlorin at indicated time points in tumours of the mice injected with Radachlorin. Figure 1(a) showed that the highest accumulation of Radachlorin in tumour was shown at 0·5 hr after injection and it was maintained for 12 hr even though its concentration was decreased to a half. Radachlorin showed a rapid clearance from sera as it is relatively long kept by tumour tissue (Fig. 1b). To see the cytotoxic effect of PDT with Radachlorin on TC-1

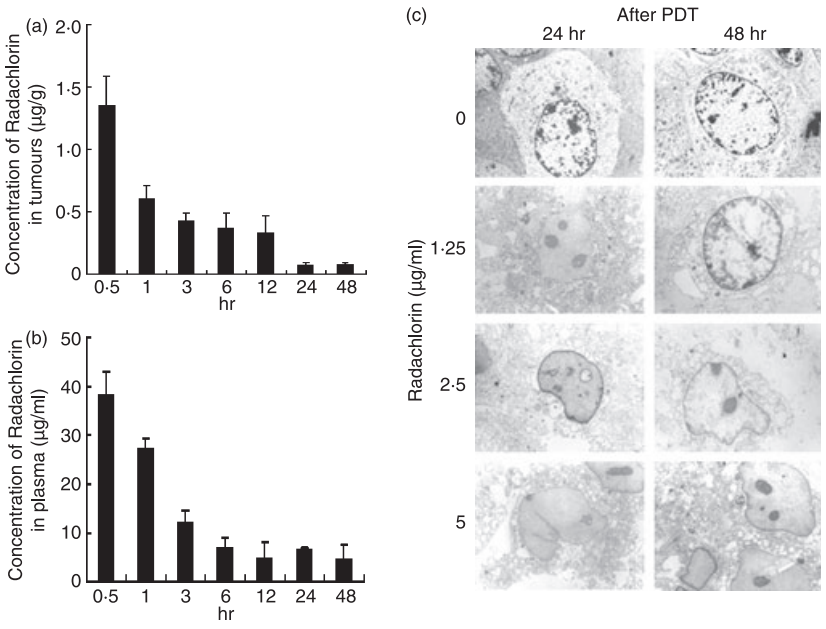

Figure 1. Concentration of Radachlorin in the tumours in mice injected with TC-1 cells. (a) Ten mice with tumours were intravenously injected with Radachlorin (10 mg/kg) followed by harvesting tumours at the indicated time points. Maximum tumour accumulation of Radachlorin was achieved at 0·5 hr. (b) Eighty percent clearance of Radachlorin from the blood was achieved after 5 hr. The bars represent the mean standard deviation (SD). (c) Representative transmission electron micrograph of TC-1 cells treated with Radachlorin/PDT. After PDT treatment with 0, 1·25, 2·5, and 5 μg/ml of Radachlorin, cells were incubated for 24 and 48 hr. The patterns of cell death shows that the plasma membrane is the target of Radachlorin, followed by yam-like processes formed around the cytoplasm, and disintegrated cytoplasm, pronounced vacuolization, shrunken condensed cytosol, nuclear chromatin condensations, lysis, plasma membrane blebbing, and extensive fragmentation of the nucleus, which confirm the loss of cell viability (TEM, magnification ×3000).

tumour cells, TC-1 cells were treated with PDT with Radachlorin and then their intracellular morphology was examined under the transmission electron microscope (TEM). While untreated TC-1 cells showed no significant morphological changes, there were drastic changes in cellular organelles after the PDT with Radachlorin (Fig. 1c). The PDT treatment against the TC-1 cells induced plasma membrane disruption and cell shrinkage, indicating Radachlorin was seen to be absorbed into the entire cytoplasm. Fluorescence image study suggested that Radachlorin could cross the plasma membrane and penetrate into mitochondria, lysosomes, and Golgi apparatus (data not shown).

The effect of PDT on *in vivo* tumoricidal activity

To see the effect of PDT on tumours, the light of 300 J/cm^2 was exposed at 0·5, 1, 3, 6, and 24 hr after Radachlorin injection (10 mg/kg) on the mice with TC-1 tumours and then tumour sizes were measured twice a week for 15 days (Fig. 2, $P < 0{\cdot}05$). Figure 2 indicated that significant differences were shown after day 10. In the PDT-untreated control group, the tumour sizes increased continually with time until the end of the observation period. PDT-treated tumours were regressed until 9 days, but PDT-treated tumours at 0·5, 1, 6, and 24 hr post-injection of Radachlorin were re-increased after 10 days. PDT treatment at 3 hr after Radachlorin injection was the most effective on both tumour regression and the inhibition of recurrence. Therefore, we determined the light dose of 300 J/cm^2 at 3 hr after Radachlorin injection for further *in vivo* experiments.

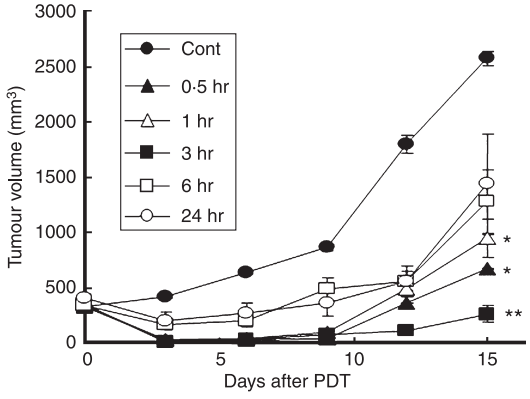

Figure 2. Influence of PDT on tumoricidal activity at different time points post-injection of Radachlorin. Tumour growth curves for TC-1 tumours after laser irradiation (300 J/cm^2) at 0·5, 1, 3, 6, 24 hr after Radachlorin injection in mice represented by filled triangle (▲), open triangle (△), filled square (■), open square (□), and open circle (○), respectively ($n = 10$ for each group). The untreated control group is indicated by filled circle (●). Statistically significant inhibition of tumour growth was measured by ANOVA. $^{*}P < 0{\cdot}05$, and $^{**}P < 0{\cdot}01$, compared with the control group.

Combination effect of PDT and AdmIL-12 on tumour suppression

IL-12 is known to enhance the cytotoxic activity of a variety of effector cells, including T cells, natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and macrophages.[17,18] It also induces the production of cytokines such as IFN-γ and subsequently, stimulates the induction of Th1 cells, which are critical for effective host defenses against intracellular pathogens. To enhance the antitumour effect of PDT, we have tried the combination therapy of PDT and AdmIL-12 on the tumours. In our observations, all the mice treated with the combination of PDT and AdmIL-12 displayed completely regressed tumour sizes over time compared with PDT or AdmIL alone (Fig. 3, $P < 0{\cdot}05$). While the effects of PDT or AdmIL-12 alone on tumour suppression gradually decreased after 10 days, the combined application of PDT and AdmIL-12 suppressed tumour growth almost completely during the experimental period.

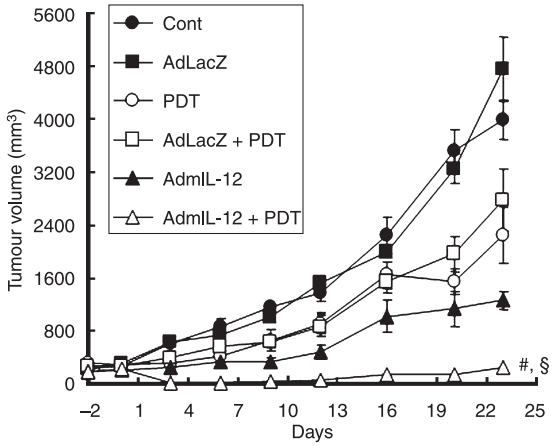

Figure 3. The effect of the combination of AdmIL-12 and PDT on tumour growth inhibition. Tumour growth curves for TC-1 of mice treated with PDT and/or AdmIL-12 ($n = 10$ for each group). Mice bearing tumours were given intratumoral injections of 1×10^8 PFU of adenoviruses expressing LacZ or IL-12. That day was designated as day −2. After 2 days, tumour-bearing mice were intravenously injected with Radachlorin (10 mg/kg). Three hr later, laser was exposed, as shown in Materials and methods. Significant inhibition of tumour growth was measured by ANOVA. $^{\#}P < 0{\cdot}05$ versus PDT alone group and $^{\$}P < 0{\cdot}05$ versus AdmIL-12 alone group. The tumour growth was significantly delayed in the group treated with AdmIL-12 plus PDT compared with the AdmIL-12 or PDT alone. Filled circle (●), untreated control; open circle (○), PDT alone; filled square (■), injection of AdLacZ alone; open square (□), injection of AdLacZ followed by PDT; filled triangle (▲), injection of AdmIL-12 alone; open triangle (△), injection of AdmIL-12 followed by PDT.

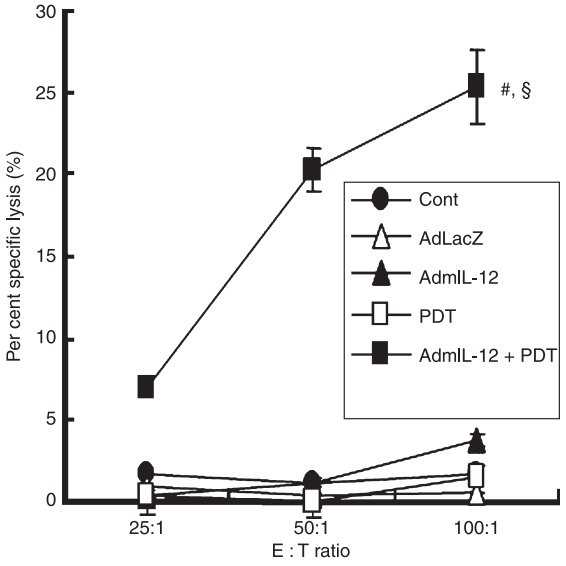

Figure 4. Induction of CTL responses by the combinatorial treatment with AdmIL-12 and PDT. Detailed methods were described in *Materials and methods*. Significant inhibition of tumour growth was measured by ANOVA. $^{\#}P < 0{\cdot}05$, compared with the PDT group and $^{\S}P < 0{\cdot}05$, compared with the AdmIL-12 group. The results represent the mean specific lysis values from individual representative mice ($n = 5$ for each group) that were tested at the indicated effector to target cell ratios (E : T ratio). The samples were assayed in triplicate. Filled circle (●), untreated control; open triangle (△), injection of AdLacZ alone; filled triangle (▲), injection of AdmIL-12 alone; open square (□), PDT alone; filled square (■), injection of AdmIL-12 followed by PDT.

The induction of CTL activity by the combination therapy of PDT and AdmIL-12

To determine whether the combination therapy of PDT and AdmIL-12 can enhance the CTL response that is an important defence mechanism against tumours, CTL activity was compared between the groups treated with PDT or AdmIL-12 alone and the combination of PDT and AdmIL-12. As shown in Fig. 4, the treatment with PDT plus AdmIL-12 elicited the CTL activity to a significant level. However, PDT alone treatment did not elicit the CTL activity even at the 100 : 1 ratio of effector to target cells (E : T ratio) and AdmIL-12 alone showed only a slight induction of CTL activity at the E : T ratio of 100 : 1. This result suggests that PDT plus AdmIL-12 dramatically enhances AdmIL-12-elicited CTL responses.

IFN-γ and TNF-α levels by the combination therapy of PDT and AdmIL-12

Because IFN-γ and TNF-α have been known to play an important role in driving Th1-type immune responses as well as inducing cytotoxic T-cell responses, we examined whether IFN-γ and TNF-α were over-secreted by the combination therapy of PDT plus AdmIL-12. We investigated the production levels of IFN-γ and TNF-α from splenocytes in mice that were treated with PDT and/or AdmIL-12. The combination therapy with PDT and AdmIL-12 dramatically increased levels of IFN-γ, compared with the other groups: i.e. AdmIL-12 or PDT alone (Fig. 5). The TNF-α level was also significantly stimulated by the combined treatment of PDT and AdmIL-12 as shown in Fig. 6.

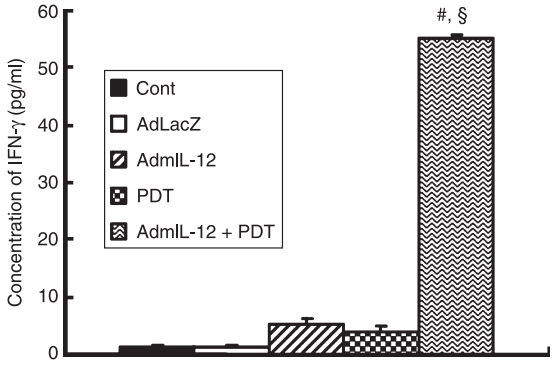

Figure 5. Dramatic induction of IFN-γ by combinatorial treatment with AdmIL-12 and PDT. Production levels of IFN-γ from splenocytes in mice ($n = 10$ for each group) that were treated with PDT and/or AdmIL-12. The samples were assayed in triplicate. Values and bars represent the mean and standard deviation of released IFN-γ concentrations, respectively. Significant inhibition of tumour growth was measured by ANOVA. $^{\#}P < 0{\cdot}05$, compared with the PDT alone and $^{\S}P < 0{\cdot}05$, compared with the AdmIL-12 alone.

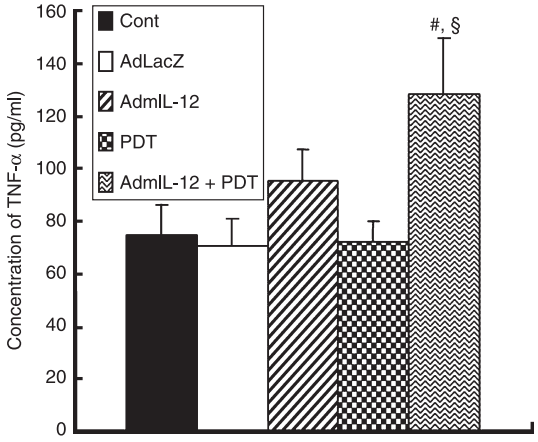

Figure 6. Production levels of TNF-α by the combination of AdmIL-12 and PDT. TNF-α level from splenocytes in mice ($n = 10$ for each group) that were treated with PDT and/or AdmIL-12 was measured. The samples were assayed in triplicate. Values and bars represent the mean and standard deviation of released TNF-α concentrations, respectively. Significant inhibition of tumour growth was measured by ANOVA. $^{\#}P < 0{\cdot}05$, compared with the PDT alone and $^{\S}P < 0{\cdot}05$, compared with the AdmIL-12 alone.

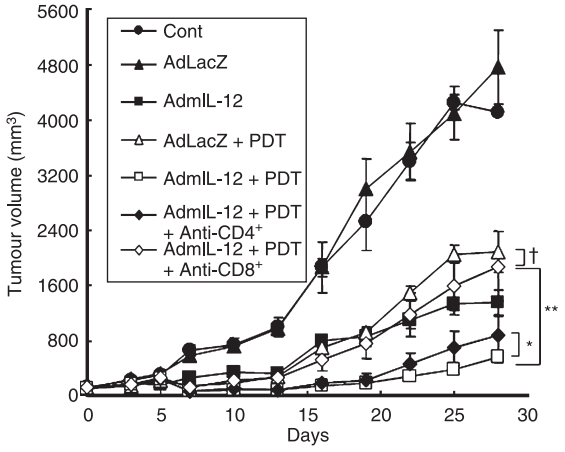

Figure 7. The Effect of T cell depletion on tumour growth inhibition by the combinatorial treatment of AdmIL-12 and PDT. The role of $CD8^+$ or $CD4^+$ T cells in mediating the antitumour response was determined using the experimental design as described in Materials and methods. Day 0 indicates the PDT-treated day. *, No statistical significance in AdIL-12 + PDT versus AdIL-12 + PDT + anti-CD4 groups (ANOVA); †, no statistical significance in AdLacZ + PDT versus AdmIL-12 + PDT + anti-CD8 groups; **, these groups (AdmIL-12 + PDT versus AdmIL-12 + PDT + anti-CD8 groups) were statistically significant ($P < 0{\cdot}05$). Filled circle (●), untreated control; filled triangle (▲), injection of AdLacZ alone; filled square (■), injection of AdmIL-12 alone; open triangle (△), injection of AdLacZ followed by PDT; open square (□), injection of AdmIL-12 followed by PDT; filled diamond (♦), AdmIL-12 and PDT and anti-CD4 ascites; open diamond (◇), AdmIL-12 and PDT and anti-CD8 ascites.

PDT plus AdmIL-12 stimulates IFN-γ production from $CD8^+$ T cells

We next evaluated whether dramatic increase in IFN-γ level by PDT plus AdmIL-12 is mediated from $CD8^+$ or $CD4^+$ T cells. As shown in Fig. 7, combined therapy of PDT plus AdmIL-12 almost completely suppressed tumour growth without immune cell depletion, which is consistent with the previous result in Fig. 3. The depletion of $CD8^+$ T cells significantly abrogated tumour suppression by the combination of PDT and AdmIL-12 while the depletion of $CD4^+$ T cells with anti-CD4 antibodies disrupted slightly the anti-tumour efficacy of PDT plus AdmIL-12. Taken together, these results indicate $CD8^+$ T cell-mediated immunity plays a pivotal role in the enhanced anti-tumour efficacy of the combination therapy of PDT and AdmIL-12.

Discussion

The present study firstly showed that antitumour efficacy of PDT plus AdmIL-12 on the animal model bearing HPV 16 E6/E7-expressed tumours was significantly enhanced, compared to that of PDT or AdmIL-12 alone. Furthermore, we also suggested here that this enhanced antitumour effect of the combination therapy be mediated by $CD8^+$ T lymphocytes and CTL response.

PDT is at present an alternative treatment of a variety of solid tumours[10,19] and recently, PDT has been used in diagnosing and treating lower genital tract carcinoma *in situ*, and advanced malignant tumours such as vulvar and ovarian carcinoma.[20,21] However, it has also shown side effects such as a small amount of damage to healthy tissue, and other temporary side effects of PDT can include coughing, trouble swallowing, abdominal pain, and painful breathing or shortness of breath. In addition, initial treatment responses after PDT are normally positive, but recurrences can occur, requiring methods to improve PDT responsiveness.[22] One reason for recurrence might be caused by the stimulation of angiogenesis by PDT treatment, suggesting the combined therapy with PDT and inhibitors. It has been reported that PDT-associated hypoxia and oxidative stress could induce the overexpression of angiogenic factor VEGF and that COX-2 expression was associated with increased activation of multiple angiogenic factors,[23,24] resulting in tumour recurrence after PDT. Combination treatment of COX-2-specific inhibitors with conventional therapies was shown to further enhance the antitumour activity of PDT in established tumours growing in mice.[25,26] These results strongly suggest that there should be trials of the combined therapy with PDT and the inhibitors of angiogenesis or COX-2 in order to enhance the antitumour efficacy of PDT in clinical trials. Also, because PDT treatment of tumour results in the rapid induction of an inflammatory response that is considered important for the activation of antitumour immunity, the combined use of proinflammatory cytokines with PDT can be expected to improve the efficacy of PDT. IL-12 stimulation plays an important role in the immune response. For example, it enhances the cytolytic activity of a variety of effector cells, including T cells, NK cells, LAK cells, and macrophages. It also induces the production of proinflammatory cytokines including IFN-γ and TNF-α. Therefore, in order to improve the tumoricidal efficacy of PDT on cancer therapy, the attempts to block the angiogenic effects of PDT or to stimulate a proinflammatory response are needed.

In this study, for the first time, we showed that the combination therapy of PDT and AdmIL-12 significantly augmented antitumour effects, compared with those of PDT or AdmIL-12 alone in a TC-1 tumour model that expresses HPV 16 E6/E7 oncogenes. It has been demonstrated that intratumoral injection of AdmIL-12 could display complete suppression against established murine adenocarcinoma and fibrosarcoma tumours as well as colon cancers.[27,28] However, we failed to detect such dramatic therapeutic effects of AdmIL-12 in the TC-1 tumour models. It can be caused by some differences in experimental environments including different cancer cell types or AdmIL-12 vectors tested here. Although AdmIL-

12 has been already used as an adjuvant for tumour immunotherapy, we showed first here the cancer therapeutic significance of the combination of PDT and AdmIL-12. Our results demonstrated that therapeutic efficacy with AdmIL-12 plus PDT resulted in a significant tumour suppression in mice bearing TC-1 tumour (Figs 3 and 7). However, treatments with either PDT or AdmIL-12 alone showed much less effect on tumour suppression, compared with that of the combination of PDT and AdmIL-12 (Fig. 3). Taken together, the combination therapy of PDT and AdmIL-12 definitely augmented therapeutic efficacy of PDT or AdmIL-12 alone in this TC-1 tumour model system.

We also evaluated CTL activities when AdmIL-12 was delivered along with PDT. Most previous reports have shown the possible effects of AdmIL-12 on the immune response.[18] Our previous report addressed that coinjection of IL-12 adenoviral vector and E7 adenoviral vector resulted in complete regression of TC-1-associated tumours, mediated by the induction of T-cell proliferation and the CTL response.[29] Coinjection with immune response modifiers plus AdmIL-12 was also reported to be capable of initiating an immune response in the absence of additional adjuvant by enhancing immunoglobulin G2a levels and CTL responses.[29] In the present study, the cotreatment with PDT plus AdmIL-12 significantly enhanced CTL activity, compared with that with PDT or AdmIL-12 alone. Furthermore, we could see a significant induction of IFN-γ production levels of Th cells. It has been reported that the proportions of cells producing IFN-γ and the amounts of IFN-γ production per cell were similar for $CD8^+$ and $CD4^+$ cells, suggesting that they both contribute significantly to IFN-γ production.[30] However, the enhancement of antitumour immunity by the combination therapy of PDT and AdmIL-12 was mediated by $CD8^+$ T cells but almost not $CD4^+$ T cells as shown in Fig. 7, suggesting that $CD8^+$ T cells are the major cell type responsible for cellular immunity in this combination therapy. In view of therapeutic application for the suppression of established tumour, effector CTL functions appear to be a critical immune parameter. There are previously published reports that IL-12 is involved in the production of IFN-γ[31] and also that IL-12 acts on T and NK cells, resulting in the production of cytokines, primarily IFN-γ.[32] These data supported the hypothesis that AdmIL-12 cotreatment with PDT can more effectively induce CTL activity, compared to that by IL-12 alone. As expected, the combination of AdmIL-12 and PDT here appeared to exert a synergistic effect on induction of CTL that includes T-cell responses (Fig. 4), resulting in cytokine production that is dominated by TNF-α and IFN-γ. Collectively, PDT plus AdmIL-12 can be useful as a therapeutic regimen against E6/E7-derived tumour cells via immune responses to IFN-γ secreting T-cell (CTL) types *in vivo*.

In conclusion, we evaluated immune responses including IFN-γ secretion and CTL activation with PDT plus AdmIL-12 injection with the TC-1 tumour challenge. We suggested that cotreatment with PDT plus AdmIL-12 could be more useful for the induction of therapeutic immunity against established HPV-associated tumour cells. Taken together, this study provides an additional option for inducing CTL-mediated antitumour therapeutic immunity against HPV-associated cervical cancer.

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