Antitumor Therapy with Bacterial DNA and Toxin: Complete Regression of Established Tumor Induced by Liposomal CpG Oligodeoxynucleotides plus Interleukin-13 Cytotoxin

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ABSTRACT

Despite urgent need, no single strategy has been widely effective at controlling the growth of rapidly progressive solid tumors. We demonstrate here a potent antitumor therapy using modified bacterial DNA and toxin. Treatment of human head and neck cancer established as xenografts in athymic mice with immunostimulatory CpG oligodeoxynucleotides encapsulated in sterically stabilized cationic liposome [(CpG ODN)SSCL] and recombinant interleukin-13 Pseudomonas exotoxin (IL13-PE) significantly reduced the tumor growth followed by complete regression in most animals. The antitumor activity of (CpG ODN)SSCL was dependent on natural killer cells that infiltrated within tumors. Interestingly, IL13-PE enhanced (CpG ODN)SSCL-induced natural killer cell activity and cytokine production in vivo and in vitro. These data strongly suggest that a combination of innate immune activation by (CpG ODN)SSCL and tumor-directed targeting by IL13-PE is a novel approach for human cancer immunotherapy.

INTRODUCTION

Despite significant advances in survival of cancer patients as a result of surgery, radiotherapy, and chemotherapy over the last century, half of patients diagnosed with invasive cancer are expected to die from it (1). Because of the urgent necessity, so-called immunotherapy has been explored as another modality for cancer therapy based on evidence that the immune system can recognize and respond to cancer cells (2, 3). One example is anticancer immunotherapy using a cocktail of two heat-killed bacteria originally demonstrated by William Coley more than a century ago (4). This initial observation of antitumor activity of bacterial extracts has often been revisited and explored by many researchers and led to the findings that components of bacteria, such as bacterial endo-/exotoxin, lipoteichoic acid, and bacterial DNA have strong antitumor activities through direct tumoricidal effects (e.g., toxin) as well as indirect effects of enhanced innate immune activation (5–9).

Genetically altered bacterial DNA contains more unmethylated CpG motifs than mammalian DNA because of CpG methylation and CpG suppression in mammalian DNA (10). Oligodeoxynucleotides containing the CpG motifs (CpG ODNs) activate B cells, natural killer (NK) cells, macrophages, and dendritic cells, resulting in robust innate immune activation (11, 12). These activities of CpG ODNs are being harnessed therapeutically in antitumor agents as well as in vaccine adjuvants and antiallergic agents (13–17). The antitumor effects of bacterial DNA and CpG ODNs have been shown in human as well as in animal models (6, 15). The activity of CpG ODNs in vivo can be further enhanced by encapsulating CpG ODNs in sterically stabilized cationic liposomes (SSCLs; Ref. 18).

Bacterial toxins such as Pseudomonas exotoxin (PE) are among the bacterial components with strong antitumor activity (9, 19). Fusion of the PE to a molecule that targets tumor cells increases cytolytic activity and reduces nonspecific cytotoxicity (20, 21). One example is IL13-PE, modified Pseudomonas aeruginosa exotoxin-A fused with interleukin-13 (IL-13), which targets PE to tumor cells expressing IL-13 receptor, such as glioblastoma, head and neck cancer, AIDS-associated Kaposi’s sarcoma, renal cell carcinoma, and prostate cancer (22–25).

Although the immunostimulatory activities of CpG ODNs have been appreciated, it has not been shown whether CpG ODNs have antitumor effects on solid tumors, such as head and neck carcinoma, or whether CpG ODNs have any impact on IL13-PE antitumor therapy if coadministered. To determine whether a combination of these two unique approaches will produce potent antitumor activity, we chose a human squamous cell carcinoma head and neck tumor model in athymic mice in which we evaluated the contribution of innate immune activation by CpG ODNs to IL13-PE-induced antitumor activity. Our study demonstrates that the combination of these two modified
“bacterial products” shows potent antitumor activity in vivo, inducing complete regression of the aggressive solid tumor in most treated mice. The mechanisms of this combination of treatments were further analyzed in vivo and in vitro.

**MATERIALS AND METHODS**

**Mice.** BALB/c athymic nude mice and NK-deficient beige mice were obtained from Frederick Cancer Research Center Animal Facilities (National Cancer Institute, Frederick, MD) and were housed under pathogen-free conditions. Animal care was in accordance with the guidelines of the Center for Biologics and Evaluation Research.

**Cells.** A human head and neck cancer cell line, KCCT873, was established in the Research Institute, Kanagawa Cancer Center (Yokohama, Japan; Ref. 25). Cells were cultured in complete RPMI 1640 containing 10% fetal bovine serum, 1 mM HEPES, 1 mM L-glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin.

**Oligonucleotides.** Synthetic single-stranded ODNs with phosphorothioate linkages were synthesized at the Center for Biologics and Evaluation Research core facility (Bethesda, MD). The sequences of the ODNs were as follows: CpG ODN (1555), 5′-GCTAGACCTAGCGT-3′; and control ODN (1612), 5′-GCTAGATGTTAGCGT-3′ (12). Endotoxin levels in all DNA stock solutions were undetectable (<0.01 unit/ml) as determined by the Limulus amebocyte lysate assay (Bio-Whittaker, Walkersville, MD).

**Preparation of SSCL.** ODNs encapsulated in sterically stabilized cationic liposomes were generated as described previously (18). Briefly, liposomes were generated by evaporating phospholipid mixtures in a round-bottomed flask on a rotary evaporator. To generate empty multilamellar vesicles, the lipid film was purged with argon. To generate small unilamellar vesicles, the lipid film was dissolved in chloroform. The chloroform was evaporated in a vacuum, and the dehydrated liposome/ODN powder and vortexed for 30 min at room temperature. PBS (900 µl) was added to the dehydrated liposome/ODN powder and vortexed for 30 min at room temperature. PBS (900 µl) was added to the mixture, yielding a final liposome concentration of 20 µmol of lipid/mg of DNA. Vesicles <150 nm in diameter were produced by 20–30 cycles of extrusion through polycarbonate filters in a Liposofast extruder (Avestin, Ottawa, Canada). Liposome formulations were stored at 4°C until use.

**Preparation of IL-13 Toxin.** The chimeric fusion gene encoding IL-13 cytotoxin (hIL13-PE38QQR; referred to as IL-13-PE) was constructed by use of human IL-13 cDNA cloned from human peripheral blood mononuclear cells and the plasmid PE38QQR (pPKL438QQR) as described previously (26, 27). Endotoxin levels were <0.01 unit/ml in all preparations.

**Human Head and Neck Cancer Xenografts, Treatments, and Evaluations.** Human head and neck tumors were established in nude or beige mice by s.c. injection of 5 × 10^6 KCCT873 cells in 150 µl of PBS plus 0.2% human serum albumin into the flank as described previously (25). Palpable tumors developed within 3–4 days. The mice then received injections of excipient (0.2% human serum albumin in PBS) or differing doses of IL13-PE by intratumoral injection (30 µl) with a 27-gauge needle. (CpG ODN)SSCL (50 µg in 20 µl) was injected in the same manner. At various time points, tumor growth was measured by Vernier calipers in a standard manner as described elsewhere (25). Tumor size was calculated by multiplying the length and width of the tumor on a given day. In some experiments, mice were rechallenged with the same number of KCCT873 cells. In some experiments, NK cells were depleted by pre- and posttreatment with rabbit anti-asialo-GM1 antibody (Ab; 50 µg/injection; Wako, Osaka, Japan) at days −3, 4, 10, and 16 after tumor implantation, whereas the control group was treated with the same amount of normal rabbit IgG (R&D Systems, Minneapolis, MN).

**Measurement of Cytotoxicity.** Mice (3 mice/group) with or without tumor implantation received intratumoral or s.c. injections of IL13-PE (50 µg/kg) with or without either (CpG ODN)SSCL or (Control ODN)SSCL (50 µg/mouse). Twenty-four h or 10 days after injection, mice were sacrificed, spleen cells were removed, and a single cell suspension was prepared. The cytotoxicities of these spleen cells against tumor cells were measured by a modified 51Cr release assay as described previously (28). Briefly, KCCT873 cells (1 × 10^6/ml) were labeled with 1 µCi of 51Cr (NEN, Boston, MA) for 18 h before the assay. Tumor cells were carefully harvested and incubated with spleen cells from treated mice as described above at various E:T ratio for 24 h. The released 51Cr in the supernatant was measured by gamma counting (Wallac Inc., Gaithersburg, MD).

**Measurement of Cytokine Production.** Supernatants from the spleen cell culture described above were immediately analyzed by ELISA to measure cytokine concentrations, as described previously (29). Briefly, 96-well Immulon 2 plates were coated with anti-asialo GM1 (BD PharMingen, San Diego, CA), or antimouse IFNγ (Biosource, Camarillo, CA) in PBS (pH 7.2) for 4 h. After the plates were blocked and washed, supernatants from stimulated cells were added and incubated for 2 h at room temperature. The plates were then washed and treated with biotinylated anticytokine Ab [IL-12 (Genzyme, Cambridge, MA) or IFNγ (Endogen, Woburn, MA)] followed by phosphatase–streptavidin (BD PharMingen). The cytokine concentration was determined by comparison with purified recombinant mouse cytokines included in the same experiment.

**Protein Synthesis Inhibition Assay.** The cytotoxic activity of IL13-PE was tested as described previously (25). Typically, 10^6 KCCT873 cells were cultured in leucine-free medium with or without various concentrations of IL13-PE38QQR for 20–22 h at 37°C, and then 1 µCi of [3H]leucine (New England Nuclear Research Products, Boston, MA) was added to each well and incubated for an additional 4 h. Cells were harvested, and the radioactivity incorporated into the cells was measured by a beta plate counter (Wallac).

**Immunohistochemistry.** Frozen sections of implanted KCCT873 tumors were prepared as described previously (30). Samples were fixed and washed with ice-cold PBS and then incubated with the following Abs diluted 1:100 in PBS containing 10% rat serum for 1 h in the dark at 4°C: anti-asialo-GM1 Ab, FITC-labeled antimouse DX-5 (pan-NK; BD PharMingen) and phycoerythrin-labeled antimouse Gr-1 or CD11b (BD
**RESULTS**

**Induction of Tumor Regression by Combination of IL13-PE Plus (CpG ODN)SSCL.** The KCCT873 human head and neck tumor cell line was used to study the effect of various therapies on tumor growth in vivo. When KCCT873 cells were implanted in the flanks of athymic nude mice, palpable tumors developed within 4 days and reached a size of \( > 200 \text{ mm}^2 \) within 4 weeks in the absence of treatment (Fig. 1A). KCCT873 cells express the IL-13 receptor \( \alpha_2 \) and thus are sensitive to treatment with IL13-PE (31, 32). Consistent with previous reports, intratumoral injection of 1 \( \mu \text{g} \) of IL13-PE into these mice (50 \( \mu \text{g/kg} \)) on days 4, 6, and 8 reduced tumor growth by \( > 75\% \) (\( P < 0.0005; \) Fig. 1A). By comparison, treating these mice with 50 \( \mu \text{g} \) of immunostimulatory CpG ODNs encapsulated in SSSL ([(CpG ODN)SSCL]) reduced tumor growth by \( > 50\% \) (\( P < 0.004; \) Fig. 1A).

Unfortunately, neither therapy alone induced complete tumor regression. Because IL13-PE and (CpG ODN)SSCL have different modes of action (the former is directly tumoricidal, the latter immunostimulatory), the effect of combining the two treatments was examined. As seen in Fig. 1A and Table 1, the combination of (CpG ODN)SSCL plus low-dose IL13-PE not only reduced average tumor size by \( > 90\% \), but led to a complete regression of tumors in 23\% of mice (\( P < 0.0005; \) Table 1). Tumors did not recur in these animals after \( > 40 \) days of follow-up (data not shown).

The effect of increasing the dose of IL13-PE was then examined. Raising the dose of IL13-PE alone was insufficient to induce complete tumor regression. Interestingly, however, when 250 \( \mu \text{g/kg} \) IL13-PE was combined with 50 \( \mu \text{g} \) of (CpG ODN)SSCL, average tumor growth was significantly reduced and complete remission was induced in 83\% of animals (Table 1; Fig. 1C). It is of note that in this tumor model the antitumor effect of IL13-PE alone was not as dose dependent as the combination treatment with (CpG ODN)SSCL, indicating that IL13-PE potentiates the antitumor activity of (CpG ODN)SSCL.

Although these tumor-free mice did not have any recurrence for as long as we observed (40 days), they accepted rechallenge of the same tumor with neither regression nor delayed growth (data not shown), suggesting that the antitumor effect of IL13-PE and (CpG ODN)SSCL was solely attributable to enhanced innate immunity.

**Histological Analysis of Tumors Treated with (CpG ODN)SSCL Plus IL13-PE.** To identify the cell types associated with tumor regression, we treated KCCT873 tumors on day 4 and removed them for histological analysis 5 and 10 days later. As seen in Fig. 2, there were considerably more CD11b

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**Statistical Analysis.** Statistical analysis was performed with the paired Student’s \( t \) test. \( P < 0.05 \) was considered significant.
granulocytes and macrophages infiltrating tumors that had been treated with IL13-PE plus (CpG ODN)SSCL than those treated with IL13-PE alone. NK cells (expressing asialo-GM1) were also present in tumors treated with combined therapy on day 5, and the number of such cells increased dramatically by day 10. In contrast, NK cells were rarely detected in untreated tumors or in tumors treated with IL13-PE alone (Fig. 2).

These data suggest that therapy with IL13-PE plus (CpG ODN)SSCL induces accumulation of macrophages and granulocytes in the peripheral regions of tumors at early phase followed by infiltration of NK cells into the tumor at late phase (day 10). IL13-PE alone induced an accumulation of macrophage and granulocytes comparable to that of control (PBS).

Antitumor Activity of NK Cells in Mice Treated with IL13-PE Plus (CpG ODN)SSCL. The above findings suggested that immune cells from mice treated with IL13-PE plus (CpG ODN)SSCL facilitated the elimination of tumor cells in vivo. To determine whether cell-mediated tumor lysis was involved, we isolated spleen cells 10 days after the last treatment and incubated them with ⁵¹Cr-labeled KCCT873 cells. Cells from untreated mice with tumors produced only low-level cytotoxicity (<20%; Fig. 3A). The spleen cells from mice treated with IL13-PE plus (CpG ODN)SSCL boosted tumor-specific cytotoxicity to >60% (P < 0.001; Fig. 3A), which was significantly higher than the cytotoxicities obtained with the controls, IL13-PE alone, or IL13-PE plus (control ODN)SSCL (P < 0.01). These data strongly suggest that (CpG ODN)SSCL increased the systemic activity of NK cells in vivo, which are then able to kill the implanted tumor.

To confirm the contribution of the increased NK activity to the antitumor effect produced by (CpG ODN)SSCL plus IL13-PE, NK cells were depleted in vivo during the treatment. To deplete NK cells, the mice received i.p. treatments of 50 μl of anti-asialo-GM1 Ab at days −3, 4, 10, and 16 after tumor implantation as described previously (33). The control group was treated with 50 μl of normal rabbit IgG. The treatment of tumor-bearing mice with anti-asialo GM1 Ab before and after the treatment with IL13-PE and (CpG ODN)SSCL abrogated the additive antitumor effect, as tumor size in animals treated with anti-asialo GM1 was comparable to the tumor size in animals treated with IL13-PE and (CpG ODN)SSCL.
treated with IL13-PE alone (Fig. 3B; data not shown). The group treated with control Ab had no change in tumor size compared with that of mice receiving IL13-PE and (CpG ODN)SSCL (Fig. 3B).

We also tested the effect of NK cells on the antitumor effect of (CpG ODN)SSCL plus IL13-PE, using beige mice lacking NK cells. KCCT873 tumor growth in beige mice was similar to growth in nude mice (Fig. 3, B and C). Treatment of the tumor with IL13-PE reduced tumor size by 43%; however, (CpG ODN)SSCL did not alter the tumor size and the number of animals showing complete remission when used alone or with IL13-PE, respectively (Fig. 3C). These data suggest that the additive effect of (CpG ODN)SSCL on IL13-PE-induced antitumor activity is dependent on NK activity.

**IL13-PE Synergizes Cytotoxicity and Cytokine Production Induced by (CpG ODN)SSCL in Vivo.** Studies were conducted to clarify the mechanism(s) involved in the antitumor effect of (CpG ODN)SSCL plus IL13-PE. Spleen cells were isolated from nude mice 1 day after treatment and tested for cytotoxicity against ⁵¹Cr-labeled KCCT873 cells (Fig. 4A). Of interest, cells from mice treated with (CpG ODN)SSCL lysed KCCT873 cells significantly more efficiently than cells from control mice or mice treated with IL13-PE alone (P < 0.05). Cells from animals treated with the combination of (CpG ODN)SSCL plus IL13-PE were even more active than those from animal treated with (CpG ODN)SSCL alone (P < 0.01), suggesting that IL13-PE might synergistically increase the ability of (CpG ODN)SSCL to activate NK cells.

IL-12 and IFNγ production by spleen cells from these animals was monitored ex vivo. Consistent with the increased NK activity observed above, cells from CpG ODN-treated mice produced significantly more of both cytokines than those from control mice or IL13-PE-treated animals (P < 0.05). The results for cells from animals treated with CpG ODN plus IL13-PE were 2–3-fold higher than those for cells from ODN-treated mice (Fig. 4B; P < 0.01). These results support the conclusion that IL13-PE synergizes with CpG ODN to activate cytotoxic and cytokine-secreting cells in vivo. On the other hand, CpG ODN had no direct effect on the viability as well as the protein synthesis of KCCT873 tumor cells in vitro, as confirmed by a protein synthesis assay (data not shown).

**DISCUSSION**

This work demonstrates that coadministration of (CpG ODN)SSCL with a tumor-specific cytotoxin leads to a pronounced reduction in tumor growth. Whereas either treatment alone decreased the rate of proliferation of the KCCT873 tumor, they are not enough to clear all tumor cells and thus lead to accumulation of macrophages and granulocytes in the tumor, which expresses IL-13 receptors (25, 27). Uptake of the cytotoxic agent by both necrosis and apoptosis (21, 34). Although these events lead to accumulation of macrophages and granulocytes in the tumor, they are not enough to clear all tumor cells and thus lead to complete regression (Fig. 2). Limiting the utility of such immunotoxins is the toxicity (particularly to the liver and...
therapy can be primarily attributed to tumor lysis plus enhanced activity of the innate immune system. Whether additional, tumor antigen-specific immunity can be induced by this combination of agents in animals with an intact immune system is the subject of an ongoing investigation. CpG ODNs have strong adjuvant-like activities and have been shown to boost the adaptive immune response to coadministered antigens (13, 14).

Recently, it has been shown that CpG ODN-induced antitumor activity can be further enhanced by other antitumor therapies, such as anti-IL-10 Ab, tumor antigen-pulsed dendritic cells in a syngeneic mouse colon carcinoma model (38, 39). Decker et al. (40) demonstrated that CpG ODNs efficiently sensitize human B-CLL cells to anti-CD25 immunotoxin by up-regulation of its target, CD25, in vitro. Our data support these observations and demonstrate that a combination of CpG ODNs and a receptor-targeted cytotoxin is superior compared to either single treatment. Thus, a combination therapy, with CpG ODNs, tumor-specific antigens, and immunotoxins could be most effective for the eradication of head and neck cancers that are resistant to conventional chemo- and/or radiation therapy.

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