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CpG-conjugated apoptotic tumor cells elicit potent tumor-specific immunity

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Abstract

The primary goal of cancer immunotherapy is to elicit an immune response capable of eradicating established tumors and preventing tumor metastasis. One strategy to achieve this goal utilizes whole killed tumor cells as the primary immunogen. Killed tumor cells provide a comprehensive source of tumor-associated antigens (TAAs), thereby eliminating the need to identify individual antigens. Unfortunately, killed tumor cells tend to be poorly immunogenic. To overcome this limitation, we covalently conjugated immunostimulatory CpG oligodeoxynucleotides (ODN) to apoptotic tumor cells and examined their ability to induce TAA-specific immune responses. Results indicate that CpG conjugation enhances the uptake of cell-based vaccines by dendritic cells (DCs), up-regulates co-stimulatory molecule expression, and promotes the production of immunostimulatory cytokines. Vaccination with CpG-conjugated tumor cells triggers the expansion of tumor-specific cytotoxic T lymphocytes (CTL) that reduce the growth of established tumors and prevents their metastatic spread. Thus, conjugating CpG ODN to cell-based tumor vaccines is an important step toward improving cancer immunotherapy.

Keywords

Cancer; Vaccine; Adjuvant; TLR9; CpG ODN

Introduction

Improving the host's ability to recognize and reject tumor cells is a key goal of cancer immunotherapy. Animal studies and clinical trials utilizing tumor-specific proteins and peptides demonstrate the feasibility of inducing an adaptive immune response capable of eradicating established tumors and tumor metastases [11, 13, 22]. Unfortunately, the success of such efforts is typically limited to vaccines targeting strong and well-defined TAAs [37].

Many tumors lack such TAAs, and consistency in the processing and presentation of tumor antigen vaccines is confounded by differences in host major histocompatibility complex (MHC) expression. Thus, the development of a widely useful strategy for inducing tumor-specific immunity remains an important goal of cancer research.

In theory, a vaccine utilizing the subject's own tumor cells should elicit a response against those TAAs best processed, presented, and recognized by that individual's immune system. In practice, whole killed tumor cell vaccines are poorly immunogenic. They not only fail to induce protective immunity but in some cases actually induce tolerance [12, 21, 24, 43]. To circumvent these problems, whole tumor vaccines have been supplemented with a variety of immune modifiers and co-stimulatory agents (including GM-CSF, IL-2, IFN γ , B7, and multiple toll-like receptor (TLR) ligands in the hope of boosting immune recognition and responsiveness [2, 3, 6–8, 28, 30, 36]. While such strategies can improve the immunogenicity of tumor cell vaccines, none has been effective in the treatment of a wide variety of tumors, tumors of clinically relevant size, and metastatic disease.

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs mimic the ability of bacterial DNA to stimulate the immune system [20]. CpG ODN trigger cells that express TLR9, promote the maturation of professional antigen-presenting cells (APCs), improve antigen presentation, trigger immunoglobulin production, and support the generation of CTL [17, 39]. It is well established that CpG ODN act as adjuvants when co-administered with vaccines targeting defined antigens (including bacterial proteins, allergens, and TAAs), where they promote both humoral and CTL responses [19, 34, 41]. Yet, simply adding CpG ODN to whole tumor vaccines fails to slow or reverse the growth of established tumors [15, 30, 38].

We and others previously documented that the efficacy of CpG-based adjuvants is critically dependent on maintaining close physical and temporal proximity between ODN and antigen (as free ODN diffuse from the site of antigen delivery) [16, 35, 40]. Physically cross-linking ODN to the target antigen can boost immunity by up to a 100-fold over that induced by simply mixing ODN with the immunogen [31, 32, 35]. This report examines whether the protective activity of whole tumor cell vaccines can be improved by chemically cross-linking CpG ODN to their surface membrane. Results show that this strategy is effective in multiple cancer models. CpG ODN conjugation improves the uptake of apoptotic tumor cells by host APCs, promotes the induction of tumor-specific responses, slows the growth of established tumors, and prevents metastatic spread. These effects were mediated specifically by CpG ODN and required the conjugation of the CpG ODN to tumor cells (as mixing free CpG with the tumor vaccine was significantly less effective). These findings suggest that CpG-conjugated autologous cancer cells show promise as immunotherapeutic agents and can be effectively combined with surgery to reduce the risk of metastasis.

Materials and methods

Animals and tumor cell lines

C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD) and studied at 6–10 weeks of age. OT-1 mice carrying ovalbumin (OVA)-T-cell receptor

transgenic T cells specific for the H-2 Kb-restricted CTL epitope on a RAG-1^{-/-} background were obtained from the Jackson Laboratory. All studies were approved by Animal Care and Use Committee (ACUC). The following cell lines were purchased from American Type Culture collection (Manassas, VA): E.G7, which is a CD8⁺ T-cell line derived from the EL-4 thymoma that had been transfected to express ovalbumin [23]; TC-1, which is a lung epithelial tumor cell line that expresses the E7 oncoprotein from human papilloma virus 16 [4]; TRAMP C-1, which is a transgenic adenocarcinoma derived from a prostate tumor that arose in a TRAMP mouse [9]; and 4T1, which is a metastatic breast cancer cell line [29].

Reagents

Phosphorothioate ODNs and NH₂ and fluorescein isothiocyanate (FITC)-labeled ODNs were synthesized at the Core Facility of the Center for Biologics Evaluation and Research, Food and Drug Administration (Bethesda, MD). The following ODNs were used: CpG ODN 1826 (TCCATGACGTTTCCTGACGTT) and control ODN 1745 (TCCATGAGCTTCCTGAGTCT). All ODN were free of detectable protein or endotoxin contamination. Endotoxin contamination was assessed using the *Limulus* amoebocyte cell lysate assay (Cambrex Bio Science, Walkersville, MD, sensitivity 0.1 units of endotoxin/mg) and protein contamination using the Pierce bicinchoninic acid protein assay kit (Thermo scientific, Rockford, IL, sensitivity 2.5 µg/ml).

Chemical conjugation of ODN to cells

The apoptosis of tumor cells was induced by adding 50 µg/ml of mitomycin C (Sigma–aldrich, St. Louis, MO) to growing cultures for 16 h at 37° in a 5% CO₂ in air incubator. Tumor cells treated with 15,000 rads could be substituted with similar effect.

Both treatments led to the apoptosis of > 90% of cells (as confirmed by annexin V and propidium iodide staining) within 24 h. Mitomycin C or irradiated tumor cells never produced tumors when transferred into naive recipients.

After the induction of apoptosis, cells were washed and incubated with 0.5–1 mg/ml of amino-modified CpG or control ODN in PBS for 1 h at 4°C, followed by 5 µM of BS3 (Thermo Scientific), a cell impermeable cross-linking agent for 1 h. Under these conditions, 70–80% of the ODN bound to the surface of apoptotic cancer cell lines (established by monitoring the loss of free ODN from cell supernatants), yielding a vaccine in which 10–20 µg of ODN was incorporated into each dose of vaccine. Unbound ODN was removed completely by centrifugation.

CFSE labeling

Tumor cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) as recommended by the manufacturer. Briefly, 2 × 10⁶ tumor cells in PBS were incubated with 0.5 µM CFSE (Invitrogen, Carlsbad, CA) for 10 min at 37°. After incubation, the cells were washed twice with PBS.

Preparation of BMDCs

Bone-marrow-derived dendritic cells (BMDCs) were prepared from C57Bl/6 mice as previously described [32]. Briefly, 10^6 cells obtained from the femur were seeded into a 100-mm Petri dish in 10 ml of RPMI supplemented with 10% fetal calf serum (FCS) and 20 ng/ml GM-CSF (BD Pharmingen, San Diego, CA). Medium was replaced on day 3, and the cells were harvested on day 7 by treatment with Trypsin/EDTA for 5 min. These cells were analyzed by FACS and found to be $> 90\%$ CD11c⁺.

Enrichment of splenic DCs

Spleens were removed under sterile conditions, cut into fragments, and incubated with RPMI 1640 supplemented with 1 mg/ml collagenase D (Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°. After washing, the cells were layered onto 50% Percoll and centrifuged for 20 min at 2,000 rpm. The interface was recovered and used as the DC-enriched fraction for flow cytometry analysis [32]. This procedure enriched CD11c cells from 2% prior to treatment to 25–30% after the enrichment.

In vivo tumor studies

Individual mice were immunized i.p. with one of the several candidate tumor cell vaccines prepared by chemically conjugating CpG ODN to apoptotic tumor cells. Mice were challenged either pre- or post-immunization with viable tumor cells s.c. (the number of cells varied with the tumor type as described in the figure legends). Tumor growth curves were generated using 5–10 mice per group, and all results were derived by combining data from 2 to 3 independent experiments. Tumor size was calculated by the formula: (length $\times$ width $\times$ height)/2. Any animal whose tumor exceeded a diameter of 2.0 cm was immediately euthanized as per ACUC protocol.

To deplete CD4⁺ vs CD8⁺ T-cell subsets, mice were injected i.p. with 25 μ l ascites of rat anti-mouse CD4⁺ (L3/T4) or mouse anti-mouse CD8⁺ (Ly2.2) Abs from Cedar-lane labs (Burlington, NC) on day -2, 0, and then twice per week post-tumor implantation.

The post-surgical model of metastatic 4T1 mammary carcinoma was described previously [29]. Briefly, mice were challenged s.c. in the flank with 5×10^4 4T1 cells. Once the primary tumor reached a diameter of > 10 mm (typically by day 14–17), it was surgically removed (including a 5-mm margin) and the mice monitored for survival.

Flow cytometry

Cells were washed with PBS, fixed in 4% paraformaldehyde for 10 min, and stained with anti-CD11c, -CD40, -CD69 or -CD86 Abs for 30 min at 4°. All Abs used were obtained from BD Pharmingen (San Diego, CA), while the PE-conjugated SIINFEKL/Kb pentamer was obtained from Proimmune (Bradenton, FL). Stained cells were washed, re-suspended in PBS/0.1% BSA plus azide, and analyzed by FACSsort (BD Biosciences). For detection of intracytoplasmic IL-12, BMDCs were co-cultured with ODN-conjugated cells and brefeldin A (Sigma-Aldrich) added 4 h later for 4 h. After staining with anti-CD11c, the cells were treated with cell permeabilization solution (BD Pharmingen) and then stained with anti-IL-12 Ab.

ELISpot assay

Single-cell suspensions were prepared from whole spleen. A total of $1.5\text{--}3.0 \times 10^5$ splenocytes/well were stimulated for 12–14 h with 100 $\mu\text{g/ml}$ of OVA (Sigma Chemical Co., St. Louis, MO) or E7 peptide (0.1 $\mu\text{g/ml}$) or apoptotic tumor cells in 96 well Immulon II plates (Millipore, Billerica, MA) previously coated with monoclonal rat anti-IFN γ Ab (R4-6A2) (BD Biosciences). The plates were washed and treated with biotinylated polyclonal goat anti-IFN γ Ab (R&D systems, MN) followed by streptavidin alkaline phosphatase. Spots were visualized by the addition of a 5-bromo-4-chloro-3-indolyl phosphatase solution (Sigma–Aldrich) in low melt agarose (Sigma–Aldrich) and counted manually under 40 $\times$ magnification. The number of cytokine-secreting cells was determined by a single blind reader, and all data were generated by analyzing three separate wells per sample.

Statistical analysis

A two-sided unpaired Student's *t* test was used to analyze tumor growth and cellular responses. The Wilcoxon's test of Kaplan–Meier plots was used to analyze differences in animal survival. *P* values <0.05 were considered to be statistically significant.

Results

CpG-conjugated tumor cells reduce the growth of established tumors

Therapeutically useful vaccines will significantly inhibit the growth of primary tumors that have reached clinically detectable size and/or prevent their metastatic spread. Initial studies examined the effect of CpG-conjugated apoptotic tumor cell vaccines on the *in vivo* growth of TRAMP C-1 cells. These tumor cells grow to form detectable masses (5–8 mm in diameter) 5–6 weeks after being injected into the flank of syngeneic C57Bl/6 mice.

The cell-impermeable cross-linking agent BS3 was used to covalently bind CpG or control ODN to the surface of mitomycin-C-treated TRAMP C-1 cells (hereafter referred to as CpG-TRAMP). Preliminary studies established that mitomycin C induced tumor cells to undergo apoptosis, that treated cells could not form tumors in naive mice, and that the conjugation procedure bound 10–20 μg of ODN per dose of vaccine to the cell surface. Substituting other methods for inducing apoptosis, such as irradiation, also produced tumor cell vaccines that were immunogenic when conjugated to CpG ODN (data not shown).

Mice were immunized and boosted with CpG-TRAMP 5–6 weeks after injecting mice with TRAMP C-1 cells (once the tumors had reached clinically detectable size). As seen in Fig. 1a, these animals generated strong cellular responses against the tumor as manifest by a significant expansion in the number of T cells responding to tumor re-stimulation *ex vivo* (*P* < 0.01, Fig. 1a).

This vaccination strategy significantly slowed the growth of established TRAMP C-1 tumors *in vivo* (*P* < 0.01 vs. unvaccinated animals, Fig. 1b). Even greater protection was achieved when CpG-TRAMP was administered weekly for 1 month (Fig. 1c). Vaccination with CpG ODN alone, unconjugated tumor cells, apoptotic TRAMP C-1 cells conjugated to control

ODN (control-TRAMP), or apoptotic TRAMP C-1 cells mixed with but not conjugated to CpG ODN (CpG + TRAMP) was ineffective at slowing tumor growth.

CpG-conjugated tumor cells prevent metastatic spread following surgical resection of primary tumors

As primary tumors increase in size and/or metastasize, cancer monotherapy is typically replaced by a combination of surgery, radiation, chemotherapy, and/or immunotherapy. Thus, the ability of CpG-conjugated tumor cell vaccines to prevent metastasis after surgical removal of primary tumors was evaluated.

4T1 mammary carcinoma cells were implanted in the flank of syngeneic mice. 4T1 cells form large primary tumors that spontaneously metastasize to the lung, liver, lymph nodes, and bone marrow [29]. After growing to > 1 cm in diameter, the primary tumor (and 5 mm of surrounding tissue) was surgically removed and the mice then vaccinated. Surgical resection eliminated the primary tumor and delayed death in control animals, but metastasis arose that were uniformly lethal (Fig. 2). In contrast, mice vaccinated post-surgery with CpG-conjugated 4T1 cells were completely protected against metastatic disease (Fig. 2). Vaccination with tumor cells alone or cells mixed with free CpG ODN did not significantly reduce metastasis or prolong life (Fig. 2).

CpG conjugation improves the immunogenicity of apoptotic tumor cells

The above results establish the utility of CpG-conjugated tumor cell vaccines as stand-alone therapy for established tumors and in preventing their metastatic spread following surgery. To clarify the immunologic mechanism(s) behind these effects, studies were conducted in the E.G7 tumor model. E.G7 tumor cells express OVA as a tumor-associated antigen, simplifying efforts to monitor the nature, speed, and specificity of the resultant immune response [23].

The ability of CpG-conjugated apoptotic E.G7 cells (CpG-E.G7) to up-regulate the expression of co-stimulatory molecules by bone-marrow-derived dendritic cells (BMDCs) was investigated. As seen in Fig. 3a, b, CpG-E.G7 induced a significant increase in the fraction of BMDCs expressing high levels of CD40 and CD86 ($P < 0.01$). This effect was not observed when BMDCs were cultured with apoptotic E.G7 cells alone or cells conjugated to control ODN (control-E.G7). CpG-E.G7 also induced CD11c⁺ DCs to produce IL-12, an effect not observed when BMDCs were cultured with control-E.G7 ($P < 0.01$, Fig. 3c, d). The amount of IL-12 induced by CpG-conjugated tumor cells was similar to that observed when BMDC were incubated with CpG ODN alone (Fig. 3b, d), although studies using FITC-labeled ODN established that no free CpG was present in the conjugated tumor cell vaccine. The activation of BMDCs by CpG-E.G7 was TLR9 dependent, since BMDCs from TLR9 KO mice failed to up-regulate co-stimulatory molecule expression or produce IL-12 (supplemental Fig. 1).

CpG-conjugated apoptotic tumor cells induce tumor-specific immunity and protect against tumor development

To examine the specificity of the vaccine-induced response, mice were immunized with either CpG-E.G7 or CpG-conjugated apoptotic TC-1 cells (CpG-TC-1) (these utilize OVA or papillomavirus E7 as their TAAs, respectively) and then challenged 21 days after immunization. As seen in Fig. 4a, d, the growth of E.G7 tumors was significantly reduced by vaccination with CpG-E.G7, while the growth of TC-1 tumors was significantly reduced by vaccination with CpG-TC-1. The protection conferred by each vaccine was tumor specific, as vaccination with CpG-E.G7 had no impact on TC-1 tumor growth and vice versa (Fig. 4b, e). Protection was abrogated by depletion of CD8⁺ but not CD4⁺ T cells, indicating that tumor-specific CD8⁺ T cells generated by CpG-conjugated vaccines were the primary mediators of tumor immunity (Fig. 4c). Tumor growth was reduced in animals vaccinated with CpG ODN mixed with (but not conjugated to) apoptotic tumor cells, but the magnitude of this effect was significantly below that induced by the CpG-conjugated vaccines and complete tumor regression never observed ($P < 0.05$).

CpG-conjugated apoptotic tumor cells induce tumor antigen-specific T cells

To further elucidate the nature of the immune response induced by CpG-conjugated apoptotic tumor cells, C57Bl/6 mice were vaccinated with CpG-E.G7 and their splenocytes re-stimulated ex vivo with OVA. Vaccination with CpG-E.G7 induced a strong Th1 cytokine response, as manifest by the OVA-dependent production of IFN γ (Fig. 5a). In contrast, E.G7 cells alone or control-E.G7 cells failed to induce antigen-specific immunity (Fig. 5a). Cells from mice vaccinated with a mixture of free CpG ODN plus E.G7 cells (E.G7 + CpG) mounted a detectable OVA-specific response that was significantly lower than that elicited by CpG-E.G7 ($P < 0.01$, Fig. 5a).

Consistent with these findings, the frequency of OVA-specific T cells identified by staining spleen cells with OVA/H2Kb pentamers was significantly greater in mice vaccinated with CpG-E.G7 vs all relevant controls (Fig. 5b and data not shown). Indeed, >2% of the CD8 T cells in mice immunized with CpG-E.G7 were pentamer-positive. This vaccination strategy also supported a significant increase in the production of OVA-specific Ab (supplemental Fig. 2), and similar results were obtained in studies involving CpG-conjugated apoptotic TC-1 cells stimulated with a CD8-specific E7 peptide (data not shown).

CpG conjugation improves the uptake of apoptotic tumor cells by DCs and triggers DCs maturation

CpG-conjugated tumor cell vaccines were significantly more immunogenic than tumor cell vaccines mixed with free ODN. To examine the mechanism underlying this improved activity, apoptotic CFSE-labeled E.G7 cells were injected i.v. and the spleens of recipient mice removed and studied by FACS. As seen in Fig. 6a, E.G7 cells conjugated to either CpG or control ODN were efficiently phagocytosed by CD11c⁺ splenocytes. The uptake of ODN-conjugated cells was twofold to threefold higher than that of unconjugated E.G7 cells ($P < 0.05$, Fig. 6b). Similarly, free CpG ODN did not improve the uptake of E.G7 cells (E.G7 + CpG).

The expression of CD69, an early marker of DCs activation, was significantly up-regulated by CD11c⁺ splenocytes that internalized CpG-E.G7 ($P<0.01$, Fig. 6c). No such effect was observed among CD11c⁺ cells that internalized unlabeled E.G7 or E.G7 cells conjugated to control ODN, nor was CD69 up-regulated in DCs that failed to internalize CpG-E.G7 or that were treated with E.G7 plus free CpG ODN (Fig. 6d).

The antigen-presenting ability of these DCs was then examined. CD11c⁺ spleen cells from vaccinated mice were co-cultured with CD8⁺ T cells from OVA-specific congenic OT-1 mice (OVA being the TAAs expressed by E.G7 cells). As expected, DCs from naive mice and mice vaccinated with E.G7 alone barely induced OT-1 cells to produce IFN γ (Fig. 7). By comparison, APCs from mice vaccinated with CpG-E.G7 triggered a sevenfold increase in the number of OT-1 cells secreting IFN γ ($P<0.001$). APCs from mice vaccinated with control-E.G7 induced a small increase in OT-1 activation, but the magnitude of this activity was significantly below that observed in mice vaccinated with CpG-E.G7 ($P<0.01$, Fig. 7).

Discussion

This work examines whether the immunogenicity and protective activity of apoptotic tumor cell vaccines are enhanced by conjugating immunostimulatory CpG ODN to their surface. Preliminary studies showed that prophylactic immunization with CpG-conjugated vaccines provided excellent protection in multiple tumor challenge models (including CT26, 4T1, B16 melanoma, LLC, and Renca tumors, data not shown). Of greater therapeutic import, the progression of established TRAMP C-1 tumors was significantly slowed, while the metastatic spread of 4T1 tumors (following surgical excision of the primary tumor) completely prevented when vaccination was initiated after the tumors had reached clinically detectable size. Mechanistic studies established that this strategy improved the uptake of tumor cell vaccines by APCs, triggered the functional maturation of the APCs, and promoted the generation of tumor-specific CTL.

Apoptotic tumor cells express a diverse array of TAAs, leading some to hypothesize that vaccines utilizing such cells would maximize the host's ability to recognize and present tumor antigens in the context of self MHC [37]. Unfortunately, whole tumor cell vaccines were found to be poorly immunogenic and could even mediate immuno-suppression [12, 21]. To overcome those limitations, we conjugated CpG ODN to the surface of apoptotic tumor cells. This strategy was premised on studies involving recombinant protein antigens showing that the adjuvant properties of CpG ODN were maximized by maintaining the ODN in close physical and temporal proximity to the immunogen [16, 35, 40]. CpG ODN directly trigger immune cells that express TLR9, initiating an innate immune response that promotes adaptive immunity. The adjuvant activity of CpG ODN has at least three components: (1) a CpG-induced enhancement in APCs function, (2) CpG-dependent induction of a cytokine/chemokine microenvironment supportive of anti-tumor immunity, and (3) improved antigen uptake mediated by DNA-binding receptors on APCs (this effect being CpG independent, and requiring that the ODN be physically linked to its target antigen) [31–33].

Mice vaccinated with CpG-conjugated vaccine were significantly better protected from tumor challenge than mice immunized vaccine plus free (unconjugated) CpG ODN or

vaccine conjugated to control ODN (Figs. 1, 2, 4). Current studies clearly demonstrated the mechanisms underlying the enhanced immunogenicity of CpG-conjugated tumor cells. CpG conjugation improved the internalization of whole tumor-based vaccines by DCs (Fig. 6). This effect was sequence independent (control ODN had the same effect), indicating that cells expressing membrane-bound ODN were more readily recognized and internalized by professional APCs. However, once internalized, the presence of CpG motifs promoted the maturation and activation of these APCs, thereby significantly improving the host's anti-tumor response (Figs. 5, 6, 7). The results indicate that essentially all of the DCs, which incorporated the CpG cell conjugate, were activated by CpG and strongly suggest that these cells would present antigenic TAAs for the preferential generation of CTLs. These findings are consistent with our and others previous studies that conjugating ODN to target proteins enhanced their uptake by APCs [31–33].

Previous studies designed to limit tumor growth by harnessing the adjuvant properties of CpG ODN failed to conjugate ODN to the target immunogen [1, 26, 42]. Rather than conjugating CpG ODN to cell-based vaccines, free ODN were co-administered with other immune modifiers (such as GM-CSF or IL-12) [30, 38]. Since free ODN are small molecules that readily diffuse from the site of injection, tumor-specific immunity was only modestly improved by those efforts. Heckelsmiller et al. co-administered free CpG ODN with apoptotic tumor cells and demonstrated that tumor-specific DCs and T cells were activated [15]. The current work shows that CpG conjugation to the tumor cell vaccine dramatically increases the magnitude of this effect. In related studies, Nierkens et al. [25] showed that CTL activity and anti-tumor efficacy required that tumor antigen and CpG ODN co-localize within the same DCs. Figure 6 confirms that observation by showing that the up-regulation of CD69 expression was limited to those DCs that internalized CpG-conjugated vaccine. Cross-linking ODN to antigen is a highly efficient method of insuring that vaccine and ODN consistently co-localize. In this context, the current study shows that CpG-conjugated vaccines are significantly more immunogenic than the same vaccine mixed with free ODN (Figs. 2, 3).

Tumor-specific CTL has a limited half-life and replicative capacity in vivo. Thus, eliminating established cancers and preventing the growth of metastasis may require the continuous induction of TAA-specific CTL. Vaccines that utilize highly immunogenic vectors to elicit tumor-specific CTL cannot be administered repeatedly, as responses against the vectors interfere with subsequent recognition of the TAAs. In contrast, CpG-conjugated tumor cells continue to stimulate new CTL and boost tumor-specific immunity when administered repeatedly (Fig. 4).

It seems unlikely that immunotherapy alone will fully eradicate most large tumors, given the enormous number of replication-competent cells present, their antigenic variability, and the presence of immunosuppressive cells in the tumor microenvironment [24]. Yet, immunotherapy may be critical to eliminating residual disease and preventing the growth of metastases after surgical resection of the primary tumor. A useful model to evaluate this possibility is provided by 4T1 mammary tumors. Similar to human breast cancer, 4T1 tumors generate frequent metastases that cause lethal disease after surgical removal of the primary tumor [29]. As seen in Fig. 2, surgery alone cannot prevent the metastatic spread of

4T1 cancer. However, immunization with the CpG-conjugated 4T1 vaccine post-surgery protected against metastatic disease. A similar effect was reported by Ohashi et al., who found that post-surgical vaccination prevented the local re-growth of murine neuroblastomas [27]. Conceptually, we imagine that effective vaccines would be prepared from fragments of the surgically resected tumors, treated to undergo apoptosis, and then conjugated to CpG ODN.

Current studies document that CpG-conjugated vaccines induce protective immunity against multiple tumor cell targets, slow the growth of established tumors, and prevent the establishment of tumor metastases (Figs. 2, 4). Since the safety of CpG-adjuvanted vaccines is well established [5, 10, 14, 18], these findings support the use of CpG-adjuvanted tumor cell vaccines for the immunotherapy of cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Treatment of established tumors with ODN-conjugated vaccine. 5×10^5 TRAMP C-1 cells were implanted into the flank of syngeneic mice on day 0. After the tumors became palpable (>5 mm in diameter at 5–6 week), the mice were vaccinated i.p. with 2×10^6 CpG or control ODN-conjugated TRAMP C-1 cells (CpG-TRAMP or cont-TRAMP), a mixture of free CpG ODN plus TRAMP C-1 cells (CpG + TRAMP) or 10 μ g of CpG ODN alone. **a** On day 60, spleen cells were isolated from unmanipulated mice (naïve), mice implanted with TRAMP C-1 cells but not further treated (No Rx) or implanted with tumor cells and vaccinated twice. These cells were then stimulated in vitro with irradiated TRAMP C-1 cells and monitored for IFN γ secretion by ELISpot assay. Results represent the mean $\pm$ SD of 6–7 mice/group from three independent experiments. **b, c** Data show the mean $\pm$ SE increase in tumor size of 6–14 mice per group derived by combining all results from 2 independent experiments ($N=14$; CpG-TRAMP 2 $\times$, $N=14$; No Rx group, $N=10$; CpG alone, $N=9$; control-TRAMP, $N=7$; CpG-TRAMP 4 $\times$, $N=6$; CpG + TRAMP). P values are in comparison with the group vaccinated 4 times. * $P < 0.05$; ** $P < 0.01$

Fig. 2.

Effect of vaccination post-surgical removal of primary tumor. 5×10^4 4T1 cells were implanted into syngeneic mice on day 0. These cells formed a tumor that was surgically removed after reaching 1 cm in diameter (typically after 2 week). The mice were then vaccinated i.p. with 2×10^6 4T1 cells mixed with or conjugated to ODN. Data show survival ($N=5$; CpG-4T1, CpG + 4T1, untreated, $N=10$; control-4T1 group). The experiment was repeated 2 times with similar results. $*P < 0.05$ (compared with all of other groups by the generalized Wilcoxon's test)

Fig. 3.

Activation of bone-marrow-derived DCs by CpG-conjugated tumor cells. **a** 5×10^5 apoptotic E.G7 cells were conjugated with $5 \mu\text{g}$ of control (*dotted line*) or CpG (*solid line*) ODN. The E.G7 cells were then cultured with 2×10^6 BMDCs for 16 h. The expression of CD40 and CD86 by DCs (unstimulated cells shown in gray) was analyzed by flow cytometry. **b** The experiment described in section (**a**) was repeated, and the fold increase in CD40 and CD86 expression was calculated by comparison with unstimulated BMDCs in each experiment. Results represent the mean + SD increase in MFI from 6 independent studies. The effect of adding $5 \mu\text{g}$ of free CpG ODN during culture is also shown. **c** 10^6 BMDCs were cultured for 8 h with 2×10^5 E.G7 cells conjugated to control or CpG ODN (control-E.G7, CpG-E.G7). Brefeldin A was added during the final 4 h of incubation. Cultures were stained to identify CD11c⁺ DCs and for the presence of intracytoplasmic IL-12. Cell frequency is shown in the upper right corner of each dot plot. All experiments were repeated three times with similar results. **d** 10^6 BMDCs were cultured for 24 h with $10 \mu\text{g/ml}$ of CpG ODN (CpG) or 2×10^5 E.G7 cells conjugated to the same amount of CpG or control ODN. The concentration of IL-12 in the supernatant of three independently studied samples per group (mean + SD) is shown. ** $P < 0.01$ versus untreated group

Fig. 4.

Prophylactic treatment of tumors with ODN-conjugated vaccines. Congenic mice were immunized i.p. with 2×10^6 apoptotic tumor cells either mixed with free CpG ODN (tumor cell + CpG) or conjugated to CpG or control ODN (CpG-tumor cell; control-tumor cell). Vaccinations were on day 21 for the TC-1 vaccine and days 42 and 21 for the EG.7 vaccine. Three weeks after the final immunization mice were challenged with 3×10^5 tumor cells and monitored for tumor growth. Data represent the mean + SE of 7–14 mice per group from 2 to 3 independent experiments. The numbers to the right of each treatment are the percent of animals/group that developed tumors. **e** The effect of depleting CD4⁺ or CD8⁺ T cells from immunized mice, as described in the methods section. * $P < 0.05$; ** $P < 0.01$ (versus the untreated group)

Fig. 5.

Induction of tumor-specific T-cell responses by ODN-conjugated vaccine. C57Bl/6 mice were immunized i.p. on days 0 and 21 with 2×10^6 apoptotic E.G7 cells mixed with or conjugated to CpG or control ODN. **a** Spleen cells were isolated on day 42, re-stimulated ex vivo with 100 μ g/ml OVA, and monitored for IFN γ secretion. Results represent the mean + SD of 5–8 mice/group from 2 independent experiments. **b** Spleen cells isolated on day 42 were stained with the SIINFEKL/Kb pentamer and analyzed by FACS to identify OVA-specific CD8 T cells. Results represent the mean $\pm$ SD of 5–7 mice/group from 3 independent experiments. * $P < 0.05$; ** $P < 0.01$

Fig. 6.

Effect of ODN conjugation on the uptake of apoptotic tumor cells by DCs in vivo. C57Bl/6 mice were immunized i.p. on days 0 and 21 with 10^7 apoptotic E.G7 cells mixed with or conjugated to CpG or control ODN. Spleens were isolated 4 h (**a**, **b**) or 12 h (**c**, **d**) later, and DCs identified and gated by staining with anti-CD11c and anti-CD69 mAbs. The uptake of CFSE-labeled E.G7 cells by CD11c⁺ DCs (monitored by FACS) is shown in **a**, and the mean + SD from four mice/group in **b**. The expression of CD69 by DCs that had internalized CFSE-labeled tumor cells is shown by histogram in **c**, while CD69 expression (MFI + SD) of 4 mice/group in **d**. Note that CD11c⁺ DCs in mice vaccinated with CpG-E.G7 that did not internalize tumor cells failed to up-regulate CD69. ** $P < 0.01$ (versus E.G7 group)

Fig. 7.

Antigen presentation by DCs isolated from vaccinated mice. C57Bl/6 mice were immunized i.p. with 10^7 apoptotic E.G7 cells mixed with or conjugated to CpG or control ODN. Spleen cells were isolated 4 h later, and 2×10^5 DC-enriched splenocytes were co-cultured with 2×10^4 OVA-expressing OT-1 cells for 24 h. Four independent experiments were performed, and the fold change in IFN γ production in each treatment group was calculated relative to the E.G7 DCs + OT-1 group in each experiment. Results represent the mean $\pm$ SD of all four experiments. * $P < 0.05$; ** $P < 0.01$