CpG Oligonucleotides Enhance the Tumor Antigen-specific Immune Response of a Granulocyte Macrophage Colony-stimulating Factor-based Vaccine Strategy in Neuroblastoma

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ABSTRACT

Granulocyte macrophage colony-stimulating factor (GM-CSF)-transduced autologous tumor cells form the basis of many immunotherapeutic strategies. We tested whether combining this approach with T-helper 1 (Th-1)-like immunostimulatory CpG oligodeoxynucleotides (CpG ODNs) would improve therapeutic efficacy in an established model of murine neuroblastoma. The weakly immunogenic Neuro-2a cell line was used in syngeneic A/J mice. CpG 1826 was tested for its antitumor effect alone and as an adjuvant to Neuro-2a cells retrovirally transduced to express murine GM-CSF (GM/Neuro-2a). Three days after wild-type (WT) tumor cell inoculation, mice in different groups were s.c. vaccinated in the opposite leg with combinations of WT neuro2a, irradiated (15 Gy) WT or GM/Neuro-2a transfectants with or without CpG 1826 (200 μg). To test for the induction of memory responses, mice that rejected their tumor were rechallenged with WT Neuro-2a (1 × 10⁶) 7 weeks after vaccination. All of the mice in the control (unvaccinated) group died within 3 weeks after Neuro-2a inoculation. Most of the vaccinated groups had only minimal-to-modest antitumor responses, and the mice succumbed to tumor. Tumor growth was remarkably inhibited in the group of mice that received irradiated GM/Neuro-2a plus CpG and four (50%) of eight mice in this group survived tumor free. Tumor-free mice were resistant to further WT tumor cell challenge, indicating a memory response. Mechanistic studies showed that CpG alone induced a favorable Th-1-like cytokine immune response and vaccine-induced tumor cell killing was dependent on both CD4 and CD8 T cells that killed tumor cell targets by apoptosis. These results demonstrate that CpG ODNs enhanced the antitumor effect of irradiated GM-CSF secreting Neuro-2a cells. This vaccine strategy elicits a potent tumor antigen-specific immune response against established murine neuroblastoma and generates systemic neuroblastoma-specific immunity.

INTRODUCTION

Neuroblastoma usually arises in the adrenal glands and is the most common extracranial solid malignancy of infancy and childhood. Although the mortality of neuroblastoma approaches 80–90% in children with the more aggressive forms of this disease, several unique forms of neuroblastoma are found in which the tumor undergoes “spontaneous” regression (1). Immune mediated apoptotic cell death is postulated as one potential mechanism explaining the spontaneous involution of neuroblastoma (2, 3). Thus, this tumor may prove to be an ideal model for studying novel immunotherapies.

Tumor vaccine development is based on the concept that host immune mechanisms can be induced to limit tumor development and destroy established tumors. Malignant tumors express TAAs and tumor-specific antigens, but effective antitumor immunity does not occur to any significant level in most tumors of advanced-stage disease. Insufficient immune stimulation and lack of host immunity may be attributable to ineffective antigen presentation. The context in which an antigen is presented to the immune system is critical for determining whether or not immune effector cells become activated. Activation of professional antigen-presenting cells occurs with activation of the innate immune system. Most innate immune defenses are normally inactive and are triggered when “pathogen-associated molecular pattern molecules” (PAMPs) or toll-like receptors are bound by particular molecules that are present in microbes but not in normal host tissues (4–7). Such stimulation induces an orchestrated series of events by activating DCs, natural killer cells, macrophages, and B cells and also by secreting multiple cytokines, chemokines, and colony-stimulating factors (8–11).

The vertebrate immune system uses toll-like receptor 9 to detect bacterial DNA based on the presence of unmethylated CG dinucleotides within particular base contexts (CpG motifs; Refs. 7, 8). The identification of CpG motifs allowed for the development of CpG ODNs that mimic bacterial DNA (12–14). CpG ODNs are potent vaccine stimulants with less toxicity than many other adjuvants (15–17) that promote the development of a Th1 response with the generation of CTLs (18, 19). DCs are antigen-presenting cells at the interface between the innate and the acquired immune system and play a key role in the modulation of immune responses by CpG ODNs. Both murine (20, 21) and human plasmacytoid DCs (22) are activated by CpG ODNs. In a previous study, it was found that maturation of DCs with CpG ODNs, induces increased IL-12 production and enhances the T-cell-stimulatory potential of the DCs in vitro (23). Furthermore, CpG ODNs enhanced the therapeutic activity of a DC-based tumor vaccine in vivo (23).

GM-CSF also plays a critical role in the maturation and function of DCs and converts Langerhans cells of the skin to immunostimulatory antigen-presenting cells. Irradiated tumor cells engineered to secrete GM-CSF stimulate potent antitumor immunity in mice (24). In a murine B-cell lymphoma model, CpG ODNs and soluble GM-CSF enhanced production of antigen-specific antibody (25). We sought to determine whether vaccinating mice with irradiated GM-CSF secreting tumor cells in the context of a CpG “danger signal,” resulted in an enhanced tumor antigen-specific immune response in a syngeneic model of neuroblastoma. This study shows that although monotherapy was only partly effective, an enhanced antitumor response was induced by combining GM-CSF and CpG and that this response was...
sufficient for the destruction of established tumors and the development of tumor-specific immunity.

MATERIALS AND METHODS

Murine Tumor Cell Lines. Neuro-2a (American Type Culture Collection, Manassas, VA) is a murine neuroblastoma derived from A/J mice and was cultured in F12/Ham’s nutrient mixture, supplemented with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum at 37°C.

Anesthetic Agents and Animal Care. Female A/J mice (6–8 weeks old; Harlan Laboratories, Indianapolis, IN) were anesthetized using halothane inhalation (Halocarbon, River Edge, NJ) during inoculation. All of the animals were housed under standard conditions in accordance with University of Iowa’s animal care and use committee, which follows the USDA guidelines for the care and use of animals. Mice were sacrificed if tumor size was greater than 3 cm in any dimension or if mice assumed a “sick mouse” posture.

CpG Inoculation. ODNs were completely phosphorothioate-modified. The following sequences were used (CG dinucleotides indicated): CpG ODN 1826: 5′-TTCATGACGT CCTCTCGAGTT-3′ (26), and the control ODN 1982: 5′-TCCAGGATCTTTCAGGTT-3′. No endotoxin could be detected in ODN preparations (<0.03 EU/μL; BioWhittaker, Walkersville, MD). ODNs were obtained from Coley Pharmaceutical Group (Welllesley, MA), diluted in PBS, admixed with the tumor cell vaccines, and administered s.c. at a dose of 200 μg/mouse.

Preparation of GM-CSF Transfectants. Glen Dranoff (Dana-Farber Cancer Institute, Boston, MA) provided MFG recombinant retrovirus encoding murine GM-CSF (24). PA317 packaging cell lines producing the GM-CSF retrovirus were generated by infecting these cells with the MFG recombinant retrovirus in the presence of Polybrene (4 μg/ml). Neuro-2a cells were then infected with retrovirus collected from PA317 supernatants. GM-CSF secreted into the medium by control, infected and irradiated Neuro-2a populations in culture were assayed by ELISA (R&D Systems, Minneapolis, MN) over a course of 9 days.

Tumor Inoculation. WT tumor cells (1 × 10^6 to 1 × 10^7) were injected into the hind leg of syngeneic mice (6–8 weeks old; Jackson Laboratory, Bar Harbor, ME). Tumor cells were washed in HBSS (Life Technologies, Inc.) and cells (volume, 200 μl) were injected s.c. Caliper measurements of tumor development and growth were documented at least every 3 days, and volumes were determined as width^2 × length × 0.52.

Vaccination. Before vaccination, Neuro-2a cells were irradiated with 15 Gy (1500 rads) using a Cesium 137 source, 1200 Ci in January 1986, (presumably 800–1000 Ci; J.L. Shephard, Model 81–16A; San Fernando, CA). Three × 10^6 irradiated tumor cells were used for vaccination.

Depletion of Effector T Cells. CD4 and CD8 T cells were depleted using the monoclonal GK-1.5 and 2D.23 antibodies, respectively (200 μg i.p. 3 days prior, on the day of, and 3 days after, the therapeutic vaccination was administered; Ref. 27). The IgG2a (SFR8-B6) isotype antibody that recognizes HLA-BW6 antigens was used as control. The efficacy of the therapeutic vaccine strategy was tested in the pre-established 3-day model after depletion of the T-cell subsets. The dosage of depleting antibody was based on previous studies, and on studies that have previously confirmed T-cell depletion (27).

RPA. The RiboQuant Multi-probe protection assay system (PharMingen, San Diego, CA) is a highly sensitive and specific method for simultaneous detection and quantification of multiple mRNA species. We customized a mouse cytokine multiprobe template set to quantify levels of the following mRNA species: TNF-α, IL-4, IL-12, GM-CSF, IFN-γ, IFN-α, IL-2, TGF-β2, L32, and GAPDH in cells of interest. (The customized probe was designed and selected to differentiate a Th1 response from a Th2 response) Radioactivity of each band in the sample is quantified and expressed as percentage L32 (ribosomal protein) of that sample.

ELISA. Standard technique with Quantitative ELISA kit from R&D systems was used to measure murine IFN-γ secreted by splenocytes cultured from vaccinated and unvaccinated mice.

Spleen Cell Preparations. Spleens were removed from mice and made into a single-cell suspension by gently working the tissue through a nylon membrane in RPMI 1640. Splenocytes were harvested for RPA analysis or cultured to detect cytokine secretion. Similarly, spleens were harvested 7 days after vaccination and T cells were enriched with SpinSep enrichment cocktail (StemCell, Vancouver, BC). CD3+ T cells were negatively separated via a density medium gradient as per manufacturer’s protocols. After enrichment, >95% purity was confirmed by FACS analysis.

Flow Cytometric Apoptosis Assay. Apoptosis is considered a major cytolytic mechanism by which T cells and natural killer cells kill targets and is, thus, a valuable end point for determining vaccine-induced immune effector mechanisms. FITC-labeled Annexin-V is used for detection of phosphotidylserine expression on early-phase apoptotic cells by flow cytometry with FACSscan (Becton Dickinson, San Jose, CA). In this assay, WT neuroblastoma target cells were cocultured with various ratios of effector T cells. Cells were resuspended in 20 μl of Annexin V-FITC (PharMingen), 20 μl of 50 μg/ml PI solution and 100 μl of incubation buffer [10 mM HEPEs/NaOH (pH 7.4), 140 mM NaCl, and 5 mM CaCl2] for 10 min. After adding an additional 400 μl of incubation buffer, cells were analyzed on FACSScan. Early apoptotic cells are those that are Annexin-V positive and PI negative, whereas dead apoptotic cells are positive for both Annexin V and PI. T cells and Neuro-2a cells were gated by size.

Statistics. The statistical analyses focused on the effects of different vaccination strategies on cancer progression. The primary outcomes of interest are time to death and tumor growth over time. Mice were given injections with tumor cells at the start of each study. The mice were then assigned to a vaccination group and were followed until death or until the experiment was terminated. The log-rank test was used to compare the median survival times between vaccination groups. Kaplan-Meier survival plots were constructed to estimate the survival functions.

Tumor sizes (cm^3) were regularly measured throughout the experiments, resulting in repeated measurements across time for each mouse. Mixed linear regression models were used to estimate and compare the group-specific tumor growth curves. Continuous first-order autoregressive structures were specified in these models to account for the within-subject correlation. A Box-Cox transformation was applied to the measured tumor sizes to stabilize the residual variance. In both the survival and growth-curve analyses, statistically significant global tests of equality across groups were followed up with pairwise comparisons to identify specific group differences. The two-tailed Student’s t test was applied to examine differences in cytokine secretion. All of the tests were carried out at the 5% level of significance. Analyses were performed with the SAS statistical software package.

RESULTS

CpG Inoculated with WT Neuro-2a Tumor Cells Inhibits Tumor Growth Locally, but Is Ineffective as a Systemic Vaccine Strategy. In preliminary experiments, we challenged mice with live WT tumor cells and CpG alone. WT neuroblastoma (Neuro-2a) in combination with CpG or control ODN (Con-1982) was administered at the site of vaccination, or the ODNs were given separately at a remote i.p. site. We found that CpG, administered locally, markedly inhibited tumor cell growth (P < 0.001), and only one of three mice developed tumor at the vaccination site (Fig. 1). Interestingly, mice that rejected tumor were not protected from a rechallenge of WT tumor cells (1 × 10^6) 6 weeks after inoculation. This observation suggests that tumor rejection induced by local CpG immune stimulation in this model failed to lead to the development of protective long-term memory. In contrast to its local protective effects, when CpG was administered i.p. at a remote site to the hind leg tumor cell challenge, the tumors appeared to grow more rapidly, and there was a trend to reduced survival.

CpG Inoculated with Irradiated Tumor Cells Inhibits Tumor Growth and Stimulates Long-Lasting Tumor Cell Immunity. In a series of studies performed evaluating the effect of tumor cell irradiation with or without CpG, no mice has ever developed tumor at the vaccination site after inoculation of irradiated Neuro-2a alone or after vaccination with irradiated cells plus CpG (15GyNeuro-2a/CpG, n > 100). All of the mice in the control group developed tumor after inoculation of WT Neuro-2a alone at a dose of 1 × 10^6 cells. Furthermore, after vaccination with 15GyNeuro-2a/CpG, mice devel-
Depletion of CD4 and/or CD8 T Cells Abrogates Antitumor Immunity. To determine the role of T cells in the observed immunity, mice were depleted of CD4+ and/or CD8+ T cells by administration of antibodies in vivo. Before depletion, mice were challenged with low-dose tumor (1 \times 10^5 cells), and subsequently, depleting antibodies were given as described in “Materials and Methods.” Mice were then vaccinated on day 3 with irradiated Neuro-2a cells plus CpG. We found that both CD4+ and CD8+ T cells were required for effective vaccination, because depletion of either T-cell subset abrogated the therapeutic effect of the vaccine (Fig. 3).

CpG Vaccination Induces a Potent Th-1 Response. Seven days after vaccination of mice with CpG alone, splenocytes were harvested from control (unvaccinated) and vaccinated mice, and mRNA cytokine profiles were evaluated by RPA. An intense up-regulation of TNF-α, IL-12, and IFN-γ was noted in the vaccinated mice (Fig. 4). Furthermore, vaccinated splenocytes secreted significantly higher levels of IFN-γ into culture media than control splenocytes (220 pg/ml versus 1500 pg/ml; \( P < 0.001 \)).

Transfected Neuro-2a Secreted Murine GM-CSF into Culture Media. GM-CSF is known to regulate differentiation of DCs for antigen uptake and presentation during induction of a cytotoxic T-cell immune response. To this end we transfected Neuro-2a with the MFG retroviral vector expressing murine GM-CSF. Neuro-2a transfectants secreted GM-CSF into culture media and after irradiation, the cells continued to secrete GM-CSF into media for up to 8 days. GM-CSF was not detected in WT controls and up to 200pg/ml GM-CSF was secreted from 1 \times 10^6 transfected cells in 24 h.

Irradiated Neuro-2a and CpG 1826 (15Gy/CpG) Vaccination Is Curative against Established Low-Dose Tumor (1 \times 10^5 Cells), but not against High-Dose Tumor (1 \times 10^6 Cells). In tumor mouse models, it is easier to obtain prophylactic protection against tumor challenge than to treat an already established tumor. For a rigorous test of this vaccine strategy, mice were inoculated with either-low dose (1 \times 10^5) or high-dose (1 \times 10^6) Neuro-2a in the right thigh, and 3 days later, the therapeutic 15Gy/Neuro-2a/CpG vaccine was administered s.c. in the left thigh. In a typical experiment, four groups of four mice each were compared: group 1 = control, low-dose/no vaccine; group 2 = low-dose/vaccine (15Gy/Neuro-2a + CpG); group 3 = control high-dose/no vaccine; and group 4 = high-dose/vaccine (15Gy Neuro-2a + CpG). Three (75%) of the four mice in group 1 died within 28 days, whereas all of the mice in the low-dose/CpG-vaccinated group rejected the pre-established tumor with 100% tumor-free survival (\( P = 0.0003 \)). All of the mice in the high-dose groups 3 and 4 died of tumor, but the growth rate was significantly slower in mice vaccinated with irradiated cells and CpG (\( P = 0.01 \); Fig. 2).

Fig. 1. Mixed linear regression model comparing tumor growth between mice inoculated with CpG 1826 or scrambled control ODNs. Group 1, CpG administered i.p. at site distant from tumor cell inoculation; Group 2, CpG admixed with tumor cells at site of inoculation; Group 3, control ODNs administered i.p. at site distant from tumor cell inoculation; Group 4, control ODNs admixed with tumor cells at site of inoculation. Tumor volume is plotted against time, and 1 \times 10^6 Neuro-2a cells were inoculated. Tumor growth was inhibited when tumor cells were inoculated with CpG at the same local site (Group 2) compared with tumor cell inoculation with either control groups 3 and 4 or CpG administration at a remote i.p. site (Group 1); \( P < 0.001 \). 75% of mice in group 2 survived tumor free.

Fig. 2. Tumor-free survival of mice inoculated s.c. with either low-dose (1 \times 10^5) or high-dose (1 \times 10^6) Neuro-2a in the right thigh. Three days later, the therapeutic 15Gy/Neuro-2a/CpG vaccine was administered s.c. in the left thigh. Four groups of four mice were compared: Group 1, control low-dose/no vaccine; Group 2, low-dose/vaccine (15Gy/Neuro-2a + CpG); Group 3, control high-dose/no vaccine; Group 4, high-dose/vaccine (15Gy Neuro-2a + CpG). Seventy-five% of mice in the low-dose control group 1 died within 28 days, whereas all of the mice in the low-dose/vaccinated group 2 rejected the pre-established tumor with 100% tumor-free survival (\( P = 0.0003 \)). All of the mice in the high-dose groups 3 and 4 died of tumor, but the growth rate was significantly slower in mice vaccinated with irradiated cells and CpG (\( P = 0.01 \); Fig. 2).

Fig. 3. Tumor growth in mice depleted of CD4 or CD8 T cells. Before depletion, mice were challenged with low-dose tumor (1 \times 10^5 cells), and, subsequently, depleting antibodies were given as described in the “Materials and Methods.” Group 1, CD8 antibody (2.43); Group 2, no depleting antibody; Group 3, CD4 antibody (GK1.5); Group 4, isotype control antibody. Mice were then vaccinated on day 3 with irradiated Neuro-2a cells plus CpG. Both CD4+ and CD8+ cells were required for effective vaccination, because depletion of either of the T-cell subsets abrogated the therapeutic effect of the vaccine. There was no difference in the growth rate of tumors in mice that received control antibody (Group 4) from those that received no antibody at all (Group 2).
mice survived long term, tumor free. GM-CSF-secreting neuroblastoma with CpG (P) had a markedly improved survival and slower growth rate compared with the other groups (Group 6 GM/Neuro 2A; Group 4 GM/Neuro 2A plus CpG). Mice that rejected their tumor were rechallenged with WT Neuro-2a (10^6) 7 weeks after vaccination. All of the mice in the control (unvaccinated) group died within 4 weeks after Neuro-2a inoculation. Similarly, mice vaccinated with GM/Neuro-2a only, irradiated GM/Neuro-2a, GM/Neuro-2a plus CpG, or irradiated WT plus CpG had minimal-to-modest antitumor responses, but all rapidly succumbed to tumor. Remarkably, tumors grew slowest in the mice that received irradiated GM/Neuro-2a plus CpG and four (50%) of eight mice in this group survived tumor free (P < 0.001; Fig. 5).

If acquired antigen-specific immune memory develops with the antitumor effects of this vaccine strategy, mice with long-term survival after tumor rejection should be protected against rechallenge with the same tumor type. Seven weeks after vaccination against established tumor, the four surviving mice were rechallenged with high-dose WT tumor. All four of the mice rejected the tumor challenge and survived tumor free.

**Activated CD3+ T cells Induce Apoptosis of Neuro-2a in Vitro.** Mice were inoculated with WT Neuro-2a, and, after 3 days, one group was vaccinated with irradiated GM/Neuro-2a plus CpG. Seven days later, CD3+ T cells were collected from both vaccinated and unvaccinated mice. CD3 + T cells were cultured for 24 h with WT Neuro-2a targets at various E:T ratios. T cells from naïve mice failed to induce apoptosis of Neuro-2a targets as determined by Annexin-V FITC labeling. T cells from vaccinated mice induced apoptosis of Neuro-2a target cells in a dose-dependent E:T ratio (Fig. 6).

**DISCUSSION**

Tumor vaccine development is dependent on the induction of host immune mechanisms for the activation of tumor-specific cytolytic effector cells. Malignant tumors express TAAs and tumor-specific antigens, but effective tumor antigen-specific immunity does not occur to any significant level in most tumors of advanced-stage disease. Insufficient immune stimulation and lack of host immunity may be partially attributable to ineffective antigen presentation.

We evaluated a novel vaccine strategy in a murine model of neuroblastoma. In this model, we used irradiated syngeneic tumor cells transduced to express GM-CSF admixed with synthetic CpG oligonucleotides in an attempt to break TAA tolerance and generate an antitumor response. We proposed that irradiated tumor cells serve as a nonproliferating vehicle for the tumor antigens. These cells were transduced to express GM-CSF to stimulate DCs and macrophages for antigen uptake and processing. We theorized that the CpG ODN admixed with the vaccine, provides a “danger signal” to the immune system (7, 8) that activates monocytes, macrophages, and DCs to express costimulatory molecules and to secrete Th1-like cytokines (28, 29).

In our neuroblastoma model, we found that CpG ODNs inoculated with viable tumor cells seemed to incite a local nonspecific immune response that destroyed tumor cells inoculated at the same site but failed to induce long-term protective immunity, perhaps because of inadequate tumor cell antigenicity. When CpG ODNs were admixed with irradiated tumor cells, a modest tumor antigen-specific immune
genes, it is apparent that GM-CSF-transduced tumor vaccines induce chemokines and cytokines in humans (15, 16 and Coley Pharmaceuticals). In an extensive analysis of tumor cell vaccine progenitor cells and converts Langerhans cells of the skin to potent immunostimulatory DCs. In conjunction with the amplified costimulatory signals (cytokines and membrane-bound ligands), the DCs engage the T-cell receptor, inducing activation and differentiation of tumor-specific cytolytic T cells capable of tumor destruction.

High-risk neuroblastoma has a dismal prognosis despite aggressive treatment with surgery and chemo- and radiation-therapy. The present data suggest that the clinical use of this immunotherapeutic vaccine strategy as an adjuvant should be investigated, to determine whether survival of children with this devastating tumor can be improved.

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REFERENCES


4 Unpublished observations.
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