CpG Oligodeoxynucleotide Enhances Tumor Response to Radiation

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Abstract

CpG oligodeoxynucleotides (ODNs) are synthetic DNA sequences containing unmethylated cytosine-guanine motifs with potent immunomodulatory effects. Via Toll-like receptor 9 agonism of dendritic cells and B cells, CpG ODNs induce cytokines, activate natural killer cells, and elicit vigorous T-cell responses that lead to significant antitumor effects, including improved efficacy of chemotherapeutic agents. On the basis of these properties of CpG ODNs, we tested whether they also could enhance tumor response to radiotherapy. Using an immunogenic mouse tumor, designated FSa, the response to radiotherapy was assayed by tumor growth delay and tumor cure rate (TCD50, radiation dose yielding 50% tumor cure rate). Treatments were initiated when established tumors were either 6 or 8 mm in diameter. CpG ODN as a single agent given s.c. peritumorally had little effect on tumor growth; however, it dramatically enhanced tumor growth delay in response to single-dose radiation by a factor of 2.58–2.65. CpG ODN also dramatically improved tumor radioresistance, reducing the TCD50 by a factor of 1.93, from 39.6 (36.1–43.1) Gy to 20.5 (14.3–25.7) Gy. The CpG ODN-induced enhancement of tumor radioresponse was diminished in tumor-bearing mice immunocompromised by sublethal whole-body radiation. Tumors treated with CpG ODN and radiation showed histologic changes characterized by increased necrosis, heavy infiltration by host inflammatory cells (lymphocytes and granulocytes), and reduced tumor cell density. These results show that CpG ODNs are potent enhancers of tumor radioresponse and as such have potential to improve clinical radiotherapy.

Introduction

Growth of malignant tumors and response to various therapeutic modalities are thought to be influenced by the immune system. Tumor development and growth may be facilitated when the immune system is deficient and may be restricted when the immune system is fully operative or stimulated (1). Similarly, conventional cancer therapies may be more effective in tumor hosts whose immune system is intact, and their therapeutic efficacy can be increased by stimulation of innate or adaptive immunity (1). In the 1970s, a number of bacteria or bacterial extracts, primarily Bacillus Calmette-Guerin and Corynebacterium parvum, were used to stimulate antitumor immunologic reactions (2). Intact bacteria and poorly characterized extracts proved to be potent stimulators of many facets of immunologic reactions. First-generation bacterial agents showed substantial activity against murine tumors but little therapeutic benefit in human clinical trials (3). An important limiting factor in their clinical application was toxicity of repeated administration (3). Active antitumor ingredients in bacterial extracts presumably were diluted and obscured by toxic contaminants such as lipopolysaccharide. Characterization and production of antitumor immunomodulators in pure form with less toxicity than the crude extract would allow additional clinical investigation.

A new way to stimulate immune function was suggested by Tokunaga et al. (4), who reported that immunostimulatory activity of Bacillus Calmette-Guerin resides in its DNA fraction and that this activity could be reproduced with synthetic oligodeoxynucleotides (ODNs). Further studies demonstrated that unmethylated CpG motifs in bacterial DNA are responsible for these immunostimulatory effects (5) and are recognized by immune cells expressing a specific receptor, Toll-like receptor 9 (TLR9; Ref. 6). In humans, only plasmacytoid dendritic cells and B cells express TLR9. By stimulating TLR9, CpG ODNs induce an activation phenotype in B cells and plasmacytoid dendritic cells characterized by expression of costimulatory molecules, enhanced antigen presentation to T cells, and secretion of T helper 1 (Th1)-promoting chemokines and cytokines (7). Because CpG-induced antigen presentation occurs in a Th1-like cytokine milieu, it stimulates development of Th1 cells and can induce high levels of cytotoxic T lymphocytes, even in the absence of T-cell help (8). Plasmacytoid dendritic cells secrete type I IFN, and CpG-activated B cells are strongly costimulated through their antigen receptor, selectively enhancing their secretion of antigen-specific antibodies (9, 10). Within hours, CpG-induced cytokines and chemokines trigger a wide range of secondary effects, such as natural killer cell and monocyte activation, which have antitumor activity.

Antitumor efficacy of CpG ODNs has been reported in a number of preventive and therapeutic tumor models (11–14). The efficacy of CpG ODN alone has been best demonstrated for small tumors, but CpG ODN also enhances cytotoxic therapy in advanced disease models (14). No studies have yet reported on the ability of CpG ODNs to improve the efficacy of radiotherapy. Therefore, this study was designed to specifically assess whether CpG ODN enhances the effect of local tumor radiation.

Materials and Methods

Mice and Tumors. C3Hf/KamLaw mice were 3–4 months old and housed four or five per cage. Animals used in this study were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture and Health and Human Services. The Institutional Animal Care and Use Committee approved all of the experimental plans and procedures. Solitary tumors of an immunogenic sarcoma (FSa) originally induced by methylcholanthrene were produced in the muscles of the right hind leg by inoculation of 5 × 10^6 cells prepared by mechanical disruption and enzymatic digestion of non-neoplastic tumor tissue.

CpG ODN. CpG ODN 1826 (sequence 5‘-TCCATGACGTTCCT/H11032-GACGTT), previously shown to be a potent stimulator of mouse TLR9 and innate and adaptive immunity, and an inactive control CpG ODN 1982 (sequence 5‘-TCCAGGACTTCTCCTCAGGT) were synthesized as described previously (13). The ODNs had no detectable endotoxin. Compounds were diluted with PBS to a concentration of 1 mg/ml and maintained at 4°C for up to 1 week. Injections were performed peritumorally in a volume of 0.1 ml to achieve a dose of 100 μg/mouse. CpG ODN 1826 was given to mice once tumors grew to 6 mm in diameter or three times, once tumors measured 6 mm, once they measured 8 mm, and again 7 days later.
Tumor Response to Radiation. The effect of CpG ODN 1826 on tumor radioresponse was determined by tumor growth delay and TCD_{50} assay (radiation dose yielding 50% local tumor cure) at 120 days. When tumors grew to 6 mm in diameter (range, 5.9–6.3 mm), mice were treated with active CpG ODN 1826. Control mice were untreated (tumor growth delay experiments) or treated with the inactive negative control CpG ODN 1982 (TCD_{50}) experiment. When tumors grew to 8 mm in diameter (range, 7.6–8.4 mm), mice were given 20 Gy single dose γ-irradiation (tumor growth delay experiments) or a range of single doses (10–55 Gy TCD_{50} experiment). Mice were given additional CpG ODN 1826 treatments at 3 h and 7 days after irradiation. For comparison, one group of mice in the tumor growth delay study was treated with only a single CpG ODN 1826 injection when the tumor reached 6 mm.

To obtain tumor growth curves, three orthogonal tumor diameters were measured at 1–3-day intervals with a vernier caliper, and the mean values were calculated. Regression and regrowth of tumors were documented until tumor diameter reached 14 mm. Tumor growth delay was expressed either as the absolute tumor growth delay in mice treated with radiation alone. In the TCD_{50} assay, mice were checked for presence of tumor in the irradiated leg at 2–7-day intervals after irradiation for up to 120 days. TCD_{50} was calculated using maximum likelihood analysis.

Whole-Body Irradiation (WBI). Tumor growth in normal mice was compared with that in mice immunosuppressed with sublethal WBI (6 Gy) 1 day before tumor cell inoculation. If the response depended solely on the ability of the host to mount an antitumor immune response, CpG ODN 1826 would be ineffective in immunocompromised mice. This approach was used in our earlier studies to suppress the effect of the host immune system on tumor response to irradiation (15). WBI was delivered 1 day before tumor cell inoculation; treatment with three doses of CpG ODN 1826 was begun when tumors grew to 6 mm; and 20 Gy single-dose local tumor irradiation was given when tumors were 8 mm in diameter.

Histologic Analysis. Tumor-bearing mice were killed by CO_{2} inhalation, and tumors were excised and placed in neutral buffered formalin. Fixed tumors were bisected along the midplane and embedded in paraffin, from which 4-μm sections were cut and stained with H&E. The percentage necrosis was determined with a Chalkley point counter using the method described by Milross et al. (16). Light-microscopic features used to identify necrosis included increased cell size, indistinct cell border, eosinophilic cytoplasm, loss or condensation of the nucleus, and associated inflammation.

Statistical Methods. Statistical analysis of tumor growth delay was performed using the SPSS v. 11.0 (Chicago, IL) program. TCD_{50} was calculated using maximum likelihood analysis. Comparison of means was carried out by t test, and differences with P < 0.05 were considered statistically significant.

Results

CpG ODN 1826 Enhanced Radiation-Induced Tumor Growth Delay. Mice bearing FSA tumors were treated with CpG ODN 1826, 20 Gy local tumor irradiation, or their combination. Treatment with CpG ODN 1826 alone delayed tumor growth for only a few days, with three injections of the agent being slightly more effective than one injection. Tumor diameter doubling time, based on growth from 6.0–12.0 mm in diameter, was increased from 8.3 ± 0.3 days in untreated mice to 10.3 ± 0.7 days (P = 0.009) after one treatment and to 12.1 ± 1.7 days (P = 0.034) after three treatments with CpG ODN 1826. Three doses of CpG ODN 1826 alone cured one of eight mice. Cured animals were excluded from analyses of tumor growth delay.

Compared with a relatively weak effect on tumor growth when given as a single treatment, CpG ODN 1826 demonstrated a strong effect on radiation-induced tumor growth delay (Fig. 1. Tumor growth delay after the combined treatment was more than the sum of tumor growth delays caused by either irradiation or CpG ODN 1826. The efficacy of irradiation was enhanced similarly by one or three doses of CpG ODN 1826, the enhancement factors being 2.65 and 2.58, respectively. However, in addition to increasing tumor growth delay, three doses of CpG ODN 1826 combined with radiation cured three of seven tumors (43%), suggesting that multiple treatments with CpG ODN 1826 were superior to a single treatment.

CpG ODN 1826 Increased Tumor Cure Rate after Radiation. To quantify the CpG ODN 1826-induced augmentation of tumor radio-responsibility, TCD_{50} assays were performed. The experimental procedure was the same as that for the tumor growth delay study, in which three doses of CpG ODN 1826 were used. Single doses of local tumor irradiation ranged from 25–55 Gy in control mice (treated with inactive CpG ODN 1982) and from 10–45 Gy in mice treated with CpG ODN 1826. Mice were observed for tumor regression and regrowth for up to 120 days after irradiation, at which time radiation dose-response curves for tumor control were calculated. As shown in Fig. 2, treatment with CpG ODN 1826 displaced the radiation response curve toward lower radiation doses (left shift), and the drug greatly reduced the TCD_{50} from 39.6 (36.1–43.1) Gy after radiation only to 20.5 (14.3–25.7) Gy (values in parentheses are 95% confidence limits). The radiation dose-modifying factor was 1.93, obtained by dividing the TCD_{50} value of the radiation-alone group with that of the CpG ODN 1826 plus radiation group.

CpG ODN 1826-Induced Enhancement of Tumor Radioresistance Was Abrogated by WBI. To test whether the enhancement of tumor radioresistance induced by CpG ODN 1826 depended on host immunocompetence, we gave mice sublethal doses of WBI. As shown in Fig. 3, tumors in WBI mice grew at a slightly slower rate than tumors in normal mice: the time to grow from 8–12 mm was 7.7 ± 0.2 days in the former and 5.3 ± 0.4 days in the latter. CpG ODN 1826 alone was similarly effective in slowing tumor growth in normal and WBI mice, producing ATGDs of 4.1 ± 0.8 days and 3.3 ± 0.6 days, respectively. Likewise, local tumor irradiation was similarly effective in delaying tumor growth in normal and WBI mice:
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Our results demonstrate a remarkable degree of synergy between CpG ODN 1826 and radiotherapy in treating large established immuno-nogenic FSa tumors. The effect was evident by increase in tumor growth delay and higher rate of tumor cure compared with radiation alone. Multiple administrations of CpG ODN 1826 were more effective than single administration. Tumor radiocurability improvement was achieved at all of the radiation doses (Fig. 2), the enhancement factor being 1.93 at the TCD50. As a single treatment, CpG ODN 1826 was only slightly effective in delaying tumor growth.

CpG ODNs have been reported to be effective for tumor immuno-therapy either alone or in combination with other therapies, including tumor vaccines, antitumor antibodies, chemotherapy, and other immunotherapies (14). As monotherapy, they have worked best when injected intratumorally or peritumorally but showed little or no activity when injected at a distant site (12, 14). In other models, CpG ODNs have induced tumor regression even when injected systemically or at a distant site (11, 13). Weigel et al. (17) recently reported that systemic CpG ODNs enhance the antitumor effects of cyclophosphamide and topotecan and improve survival after surgical resection of murine rhabdomyosarcoma. Although neither cyclophosphamide nor CpG ODN alone was curative, combination treatment resulted in long-term survival of 15–40% of mice.

Even in the absence of a vaccine, there is reason to hypothesize that CpG ODN induces an antigen-specific antitumor T-cell response. Injection of CpG ODN creates a Th1-like cytokine/chemokine milieu within the enlarging lymph nodes are large numbers of dendrite cells and lymphadenopathy in the draining lymph nodes (18). Among cells within the enlarging lymph nodes are large numbers of dendrite cells and granulocytes and contained large areas of reduced tumor cell density (Fig. 4, C and D).

Discussion

ATGDs were 7.8 ± 1.7 days and 6.9 ± 0.7 days, respectively. In contrast, the effect of CpG ODN 1826 on tumor radioreponse was different in the two groups. In normal mice, CpG ODN 1826 strongly augmented tumor radioreponse, as evidenced by a 50% tumor cure rate and a significant increase in radiation-induced growth delay of tumors that did not regress. Conversely, in WBI mice, CpG ODN 1826 caused no tumor cure nor did it increase radiation-induced tumor growth delay (Fig. 3).

CpG ODN 1826-Induced Tumor Cell Depopulation, Necrosis, and Host Cell Infiltration. Histology of FSa tumors from animals treated with CpG ODN 1826 when 6 and 8 mm in diameter and irradiated (20 Gy) when 8 mm in diameter was compared with those not treated with CpG ODN 1826 or radiation and with those treated with CPG ODN 1826 or radiation as single agents. Untreated tumors were highly cellular, contained small areas of necrosis (median, 4.8%), and showed little evidence of host cell infiltration (Fig. 4A). Six days after CpG ODN 1826 treatment, tumors showed similar histologic appearance to that of untreated tumors with the exception of an increased percentage of necrosis (median, 26.7%). Tumors treated with radiation only also were more necrotic (median, 14.8%) than untreated tumors, but they also exhibited extensive cellular polymorphism typically associated with radiation, including the presence of large multinucleated cells, polyploid cells, and cells with large swollen nuclei (Fig. 4B). Tumors treated with CpG ODN 1826 and radiation also were more necrotic (median, 27.9%) than untreated tumors, and compared with tumors exposed to radiation only, they showed more prominent radiation-induced cellular polymorphism. These tumors also were heavily infiltrated with host mononuclear cells and granulocytes and contained large areas of reduced tumor cell density (Fig. 4, C and D).

Fig. 2. Effect of CpG oligodeoxynucleotide (ODN) 1826 on tumor radiocurability. The percentage of tumor cures was plotted as a function of radiation dose. Mice bearing FSa tumors in the leg were exposed to a range of single radiation doses when 8 mm in diameter, and they were treated three times with the active CpG ODN 1826 (●) or the inactive CpG ODN 1982 (○) at a dose of 100 μg per mouse given s.c. peritumorally when tumor diameters were 6 mm and 8 mm and 1 week later. The TCD50 (dose of radiation needed to produce 50% tumor cures in irradiated mice) was determined 120 days after irradiation. TCD50 values and their 95% confidence limits were 39.9 (36.1–43.1) and 20.5 (14.3–25.7) Gy for irradiated mice treated with the inactive control and with active CpG ODN 1826, respectively. The horizontal bars at the TCD50 are 95% confidence limits. The radiation dose-modifying factor was 1.93 (obtained by dividing TCD50 value of the radiation plus negative control group with that of the radiation plus active CpG ODN 1826 group).

Fig. 3. Effect of CpG oligodeoxynucleotide (ODN) 1826 on radiation-induced FSa tumor growth delay in normal (top) and whole-body irradiation (WBI) mice (bottom). Normal and WBI mice bearing FSa tumors in the leg were untreated (○) or treated with CpG ODN 1826 three times (●), 20 Gy local tumor irradiation (◇), or three doses of CpG ODN 1826 plus radiation (●). Irradiation was delivered when tumors were 8 mm in diameter. Treatment with CpG ODN 1826 at a dose of 100 μg per mouse was given s.c. peritumorally when tumors were 6 mm and 8 mm and 1 week later. Groups consisted of 10 or 11 mice each. However, 5 of 10 normal tumor-bearing mice treated with CpG ODN 1826 plus radiation were cured and excluded from the tumor growth delay analysis; bars, SE.
Fig. 4. Histologic appearance of FSa tumors, untreated (A) or treated with 20 Gy local tumor irradiation (B) or treated with a combination of CpG oligodeoxynucleotide (ODN) 1826 plus irradiation (C and D). CpG ODN 1826 (100 μg s.c. peritumorally) was given when tumors were 6 mm and 8 mm in diameter, and 20 Gy local tumor irradiation was delivered when tumors were 8 mm in diameter. Histologic sections were obtained 6 days after tumor irradiation and last dose of CpG ODN 1826 and stained with H&E.

that express increased levels of costimulatory molecules and MHC, peaking at ~7–10 days after injection (18). CpG ODN activation of dendrite cells promotes strong memory T-cell responses (14). CpG ODN-primed mice respond to subsequent antigen injection in the same anatomic region with a strong Th1-based response and high levels of cytotoxic T lymphocytes even several weeks after the CpG ODN injection (18). We hypothesize that when radiotherapy is given following CpG ODN injection, tumor antigens that are released from dying tumor cells are taken up and presented by activated dendrite cells, leading to the induction of a tumor-specific T-cell response. The curative efficacy of this combination therapy was lost in mice that had been rendered immunoincompetent by WBI, consistent with our hypothesized mechanism of action. WBI did not appear to eliminate the modest effect of CpG ODN monotherapy because these mice still showed some CpG ODN-induced tumor growth delay regardless of whether the tumor was irradiated. It is possible that this modest effect of CpG ODN alone results from activation of innate immune effector mechanisms, possibly less sensitive to WBI.

Earlier studies showed that the radioresponse of FSa tumors was strongly enhanced by treatment with corynebacteria (2, 15, 19). Human clinical trials of C. parvum were initiated but showed a disappointing lack of efficacy (3). The radioenhancing efficacy of CpG ODN in our current experiments is at least as high as that previously demonstrated for bacterial extracts. Early human clinical trials reveal that CpG ODN is well tolerated and highly immunostimulatory, even after repeated weekly dosing for >1 year (20). On the basis of these results, pure chemical TLR9 agonist CpG ODNs are major milestones in rational immunomodulator development and are promising agents for additional clinical investigation in combination with radiotherapy. Further studies are needed to assess whether CpG ODNs enhance radioresponse of nonimmunogenic tumors and whether they affect radiation-induced normal tissue damage.

References


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