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Targeting Toll-like Receptor 9 with CpG Oligodeoxynucleotides Enhances Tumor Response to Fractionated Radiotherapy

Kathryn A. Mason,¹ Hisanori Ariga,¹ Robert Neal,¹ David Valdecanas,¹ Nancy Hunter,¹ Arthur M. Krieg,² John K. Whisnant,² and Luka Milas¹

¹Department of Experimental Radiation Oncology, University of Texas M.D. Anderson Cancer Center, Houston, Texas and ²Coley Pharmaceutical Group, Inc., Wellesley, Massachusetts

ABSTRACT

Synthetic oligodeoxynucleotides containing unmethylated CpG motifs detected by Toll-like receptor 9 of dendritic cells and B cells have potent immunomodulatory effects. CpG oligodeoxynucleotides induce cytokines, activate natural killer cells, and elicit T-cell responses leading to antitumor effects, including improved efficacy of chemotherapeutic agents and, as we reported recently, synergy between CpG oligodeoxynucleotide 1826 and single-dose radiotherapy of an immunogenic mouse fibrosarcoma. The present study extends this finding to the fractionated radiotherapy of the fibrosarcoma tumor and assesses the ability of CpG oligodeoxynucleotide 1826 to increase the radioresponse of a tumor (nonimmunogenic fibrosarcoma). The experiments used a murine immunogenic fibrosarcoma tumor, fibrosarcoma growing in the leg of mice, and response to radiotherapy was assessed by tumor growth delay and tumor cure rate (TCD₅₀, radiation dose yielding 50% tumor cure). Multiple s.c. peritumoral or i.t. administrations of CpG oligodeoxynucleotide 1826 at a dose of 100 µg per mouse were given when established tumors were 6 mm in diameter. Local tumor irradiation was initiated when tumors grew to 8 mm in diameter; radiation was delivered in 1 to 9 Gy fractions given twice daily separated by 6 to 7 hours for 5 consecutive days to achieve a total dose of 10 to 90 Gy. CpG oligodeoxynucleotide 1826, given as a single agent, had only a small antitumor effect, but it dramatically enhanced fibrosarcoma response to radiotherapy. Although 83.1 (79.2-90.0) Gy total dose were needed to achieve tumor cures in 50% of mice treated with radiotherapy alone, only 23.0 (11.5-32.7) Gy total dose were needed in mice treated

with both CpG oligodeoxynucleotide 1826 and radiotherapy. The magnitude of potentiation of tumor radioresponse at the TCD₅₀ level was by a factor of 3.61, a much higher value than that (a factor of 1.93) that we reported for single-dose radiotherapy. Mice cured of their tumors by combined CpG oligodeoxynucleotide 1826 plus radiotherapy were highly resistant to s.c. tumor take or development of tumor nodules in the lung from i.v. injected tumor cells when rechallenged with fibrosarcoma cells 100 to 120 days after the treatment, suggesting the development of a memory response. CpG oligodeoxynucleotide 1826 also increased radioresponse of the nonimmunogenic fibrosarcoma tumor by a factor of 1.41 and 1.73 when CpG oligodeoxynucleotide 1826 was given s.c. and i.t., respectively. These findings show that CpG oligodeoxynucleotides are highly potent enhancers of tumor response to both single-dose and fractionated radiation and as such have potential to improve clinical radiotherapy.

INTRODUCTION

The immune system plays an important role in tumor development and growth and in tumor response to conventional therapeutic treatments (1). Immune deficiency is, in general, conducive to tumor growth and lowers tumor response to therapy; stimulation of the immune system may result in the opposite outcome. Consequently, immunologic approaches for cancer treatment either as single-modality therapy or in combination with conventional cancer treatments have been developed and used.

One immunotherapeutic approach has been to stimulate antitumor immunologic reactions of the tumor-bearing host by using bacteria or bacterial extracts, such as *Bacillus Calmette-Guerin* and *Corynebacterium parvum* (2, 3). These bacteria or their extracts are potent elicitors or augmentors of many features of immunologic reactions, including activation of macrophages, induction of natural killer cell lytic activity, induction of antibody-dependent cell cytotoxicity, and production of various cytokines with antitumor activity, such as IFN and tumor necrosis factor- α . They were also shown to be very potent against various types of tumors in rodents and to improve the efficacy of chemotherapy and radiotherapy (2, 3). In contrast to these preclinical results, however, clinical use of these bacterial agents had only modest therapeutic benefit (4). It should be noted that the full exploration of therapeutic potential of whole bacteria or their crude extracts in the clinic was hampered by severe toxicity associated with repeated administration.

The discovery that the immunostimulatory activity of bacteria resides in their DNA offered the prospect for significant improvement in tumor immunotherapy (5). It was further established that the immunostimulatory activity of bacterial DNA resides in DNA unmethylated CpG motifs (6) that are prevalent in bacterial but not in vertebrate genomic DNA.

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Requests for reprints: Luka Milas, Department of Experimental Radiation Oncology-66, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009. Phone: 713-792-3263; Fax: 713-794-5369; E-mail: lmilas@mdanderson.org.

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Consequently, oligodeoxynucleotides containing unmethylated CpG motifs were synthesized and tested. Different bacterial components or bacterial products, such as lipopolysaccharides, proteoglycans, flagellin, and endotoxin as well as CpG motifs, are recognized by the innate immune cells through Toll-like receptors (TLR). There are 10 different TLR, but only TLR9 of plasmacytoid dendritic cells and B cells recognize CpG oligodeoxynucleotide, a signaling pathway resulting in lower toxicity compared with the other TLR signaling pathways (7). In mice but not in humans, TLR9 is also expressed in myeloid dendritic cells and monocytes (8–10). Most other TLR activators are toxic systemically.

CpG oligodeoxynucleotides are potent stimulators of both innate and adaptive immunologic responses. They activate plasmacytoid dendritic cells and B cells, which then acquire an increased ability to present antigens to T cells and to secrete different cytokines and chemokines that, within several hours after CpG oligodeoxynucleotide administration, trigger a wide range of secondary effects, such as natural killer cell and monocyte activation, which have antitumor activity (11). This early innate immune response is followed within several days by induction of the adaptive immune response characterized by high levels of CTL (12, 13) and antigen-specific, antibody-producing B cells (14–16).

Increasing evidence in experimental animals shows that CpG oligodeoxynucleotides exert antitumor activity against different types of tumors in both preventive and therapeutic settings. The effect has been observed mainly when the treatment was initiated when the tumors were small, and the effect was manifested usually as a delay in tumor growth and prolongation in tumor-host survival (17–24). Treatment with CpG oligodeoxynucleotides has also been reported to improve the outcome of surgery (23, 24), chemotherapy (23, 24), and, most recently, radiotherapy (25).

Our recently reported initial study showed that CpG oligodeoxynucleotide 1826, non-antisense DNA sequences containing unmethylated CpG motifs and a nuclease-resistant phosphorothioate backbone, was highly potent in enhancing the response of the immunogenic mouse sarcoma (fibrosarcoma) to single-dose local tumor irradiation (25). CpG oligodeoxynucleotide 1826 enhanced radiation-induced tumor growth delay by a factor of >2.5 and of tumor radiocurability by a factor of ~2.0. Tumors treated with both CpG oligodeoxynucleotide and radiation were heavily infiltrated by host inflammatory cells (lymphocytes and granulocytes) and showed histologic changes characteristic of massive tumor cell destruction, including increased necrosis and reduced tumor cell density. The CpG oligodeoxynucleotide-induced enhancement of tumor radioresponse was diminished in tumor-bearing mice immunocompromised by sublethal whole-body radiation, demonstrating that the antitumor efficacy of CpG oligodeoxynucleotide required the presence of an intact immune system.

To assess therapeutically relevant interactions between CpG oligodeoxynucleotides and of a clinically relevant technique of radiotherapy, we combined CpG oligodeoxynucleotide 1826 with fractionated radiotherapy to treat mice with fibrosarcoma implants. We measured tumor growth delay and tumor cure. We also asked whether regression of tumors as a

result of the combined treatment resulted in host resistance to tumor cell rechallenge. In addition, we tested whether CpG oligodeoxynucleotide 1826 can enhance the radiation response of a tumor.

MATERIALS AND METHODS

Mice and Tumors. Male C3Hf/KamLaw mice bred and maintained in our own specific pathogen-free mouse colony were 3 to 4 months old at the beginning of the experiments 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 U.S. Department of Agriculture and Department of Health and Human Services. Most experiments were done using an immunogenic sarcoma, designated fibrosarcoma, originally induced by methylcholanthrene in this strain of mice (26). For these studies, the tumor was in its 6th isograft generation. We also tested the ability of CpG oligodeoxynucleotide 1826 to enhance the radiation response of a sarcoma, designated nonimmunogenic fibrosarcoma (27, 28). This tumor spontaneously arose in this strain of mice and was in its 11th isograft generation when used in the present study. Solitary tumors were produced in the muscles of the right hind leg by the inoculation of 5×10^5 cells. Tumor cell suspensions were prepared by mechanical disruption and enzymatic digestion of non-necrotic tumor tissue (29).

CpG Oligodeoxynucleotide. The active CpG oligodeoxynucleotide 1826 (sequence TCCATGACGTTCCCTGACGTT) had a nuclease-resistant phosphorothioate backbone and was provided by Coley Pharmaceutical Group, Inc. (Wellesley, MA). A control oligodeoxynucleotide with no CpG motif, oligodeoxynucleotide 2138 (sequence TCCATGAGCTTCCTGAGCTT), was used as an inactive negative control. The compounds were diluted with PBS to a concentration of 1 mg/mL and maintained at 4°C for up to 1 week. Injections were done peritumorally or i.t. in a volume of 0.1 mL to achieve a dose of 100 µg per mouse. Treatment with CpG oligodeoxynucleotide 1826 was begun when tumors were 6 mm in diameter. The mice that received CpG oligodeoxynucleotide 1826 as the only treatment were given 100 µg of the agent per mouse thrice: once tumors measured 6 mm, once they measured 8 mm, and again 7 days later. When the agent was combined with tumor irradiation, it was given either in three doses as described above or in seven doses where the four additional doses of CpG oligodeoxynucleotide 1826 were given on a weekly basis. This 6-week overall treatment time was selected to mimic the duration of treatment commonly used for clinical radiotherapy regimens.

Tumor Irradiation. Radiation treatments were begun when tumors were 8 mm in diameter. Unanesthetized mice were immobilized in a jig, and tumors were centered in a 3 cm diameter circular field. Radiation was delivered to the tumor-bearing leg using a dual-source ^{137}Cs γ -ray unit at a dose rate of 5.5 Gy/min. The tumor-bearing mice were treated with CpG oligodeoxynucleotide 1826 or local tumor irradiation plus the inactive negative control (CpG oligodeoxynucleotide 2138) alone or with local irradiation 3 hours before the second of

seven doses of CpG oligodeoxynucleotide 1826. In tumor growth delay experiments, radiation was delivered for 5 consecutive days in 2 Gy fractions (total dose 20 Gy) given twice daily separated by 6 to 7 hours or as a single dose of 20 Gy. For the TCD₅₀ (radiation dose yielding 50% tumor cure) assay, only fractionated local tumor irradiation was used as described above. The fractional doses ranged from 2 to 9 Gy to achieve total doses of 20 to 90 Gy in control mice and from 1 to 6.5 Gy to achieve total doses of 10 to 65 Gy in mice treated with seven doses of CpG oligodeoxynucleotide 1826.

To obtain tumor growth curves, three orthogonal tumor diameters were measured at 1- to 3-day intervals with a Vernier caliper, and the mean values were calculated. Regression and regrowth of tumors were followed until tumor diameter reached ~14 mm at which time the mice were euthanized. Tumor growth delay was expressed as either absolute tumor growth delay (ATGD) or normalized tumor growth delay (NTGD). ATGD was defined as the time (in days) for tumors in the treatment arms to grow from 8 to 12 mm in diameter followed by sustained tumor growth minus the time (in days) for the tumors in the untreated control group to reach the same size. NTGD was defined as the time (in days) for tumors in groups treated with a combined regimen to grow from 8 to 12 mm minus the time (in days) to reach the same size in mice treated with CpG oligodeoxynucleotide 1826 alone.

In the TCD₅₀ assay, mice were checked for the presence of tumor in the irradiated leg at 2- to 7-day intervals after irradiation for up to 100 days. A total of 86 mice were treated with irradiation plus active CpG oligodeoxynucleotide 1826, and a total of 74 mice were treated with irradiation plus negative CpG oligodeoxynucleotide 2138.

Resistance of Cured Mice to Rechallenge with Tumor Cells. Mice cured of their fibrosarcoma tumors by either local tumor irradiation only or combined CpG oligodeoxynucleotide 1826 plus tumor irradiation were tested for their ability to resist tumor development when rechallenged with fibrosarcoma cells. Mice were challenged with tumor cells using either s.c. or i.v. routes of injection 100 to 120 days after initial radiation treatment. Tumor growth in cured mice was then compared with that for aged-matched normal mice. In s.c. rechallenge, mice were given s.c. injections in two to four sites overlying the abdomen of 1.25×10^4 to 8.0×10^5 viable tumor cells. Thereafter the mice were examined twice to thrice weekly for tumor development for up to 60 days after tumor cell transplantation. To produce tumor nodules in the lung, mice were given i.v. injections of 2.5×10^4 to 1.2×10^6 fibrosarcoma tumor cells. Mice were killed 14 days after tumor cell injection, their lungs were removed, and lung lobes were separated and fixed in Bouin's solution. Colonies of tumor cells (artificial metastases) appeared as white, round nodules on the surface of the yellowish lung and were counted with the naked eye.

Statistical Methods. Statistical analysis was done using SPSS V11.0 (Novell). Comparison of the tumor growth delay means was carried out by *t* test; the TCD₅₀ was calculated using maximum likelihood analysis. Comparison of median values of lung tumor nodules was carried out by Mann-Whitney test. Differences with *P* < 0.05 were considered statistically significant.

RESULTS

Effect of CpG Oligodeoxynucleotide 1826 on Tumor Growth Delay by Single-Dose or Fractionated Radiation.

Mice bearing fibrosarcoma tumors were treated with CpG oligodeoxynucleotide 1826 three or seven times, single-dose or fractionated local tumor irradiation, or both CpG oligodeoxynucleotide 1826 and radiation. The results in Table 1 and partly in Fig. 1 show that a single dose of 20 Gy radiation caused tumor growth delay of 6.9 ± 0.8 days (Table 1, group 3) or 7.8 ± 2.6 days (Table 1, group 7), whereas the same total dose of radiation given in ten 2 Gy fractions produced an ATGD of only 1.1 ± 0.3 days (Table 1, group 5) or 1.4 ± 0.2 days (Table 1, group 9). The lower antitumor efficacy of 20 Gy given in fractional doses of 2 Gy compared with a single dose of 20 Gy can be attributed to the well-established radiobiological factors of radiation damage repair and repopulation of tumor cell clonogens between radiation fractions (30).

CpG oligodeoxynucleotide 1826 alone delayed tumor growth for only a few days (ATGD = 3.1 ± 0.4 ; *P* < 0.0001), which is consistent with our earlier findings (25). In contrast, CpG oligodeoxynucleotide 1826 combined with 20 Gy single-dose radiation was strongly effective when given both three and seven times. The combination of three doses of CpG oligodeoxynucleotide 1826 with radiation cured 6 of 10 (60%) tumor-bearing mice and greatly delayed the growth of tumors in the remaining four mice. The ATGD of these remaining tumors was 20.3 ± 0.8 days, considerably more than the sum of tumor growth delays caused by radiation (6.9 ± 0.8 days) or CpG oligodeoxynucleotide 1826 (3.1 ± 0.4 days), suggesting synergy between the two agents. The resulting radiation enhancement factor was 2.49. Similarly, more additivity was obtained when seven doses of CpG oligodeoxynucleotide 1826 were combined with 20 Gy local tumor irradiation. The combined treatments resulted in 5 of 11 (45%) mice cured and in ATGD of 44.1 ± 7.7 days for tumors in mice not cured. This ATGD value was considerably higher than the sum of tumor growth delays caused by individual treatments, 7.8 ± 2.6 days by irradiation and 3.1 ± 0.4 days by CpG oligodeoxynucleotide 1826. The enhancement factor was 5.26.

Treatment with either three or seven doses of CpG oligodeoxynucleotide 1826 also enhanced tumor radioresponse to fractionated irradiation in terms of both increasing tumor growth delay and inducing a high percentage of tumor cure. Three doses of CpG oligodeoxynucleotide 1826 combined with 20 Gy fractionated irradiation resulted in 4 of 10 (40%) mice cured and in ATGD of 21.1 ± 7.6 days for tumors in mice not cured. This ATGD value was higher than the sum of tumor growth delays caused by individual treatments, 1.1 ± 0.3 days by irradiation and 3.1 ± 0.4 days by CpG oligodeoxynucleotide 1826 (enhancement factor = 16.36). Seven doses of CpG oligodeoxynucleotide 1826 combined with 20 Gy fractionated irradiation resulted in 4 of 11 (36%) mice cured and in ATGD of 14.1 ± 5.9 days for tumors in mice not cured. This ATGD was higher than the sum of tumor growth delays caused by individual treatments, 1.4 ± 0.2 days by irradiation and 3.1 ± 0.4 days by CpG oligodeoxynucleotide 1826 (enhancement factor = 7.86). Thus, compared with the results after single-dose irradiation, CpG oligodeoxynucleotide 1826 causes a similar rate of tumor cure.

Table 1 Effect of CpG oligodeoxynucleotide 1826 on response of fibrosarcoma tumors to single-dose and fractionated radiation

| Group and treatment* | Cures/total mice† | Tumor growth delay (mean ± SE) | | | | Enhancement factors |
|--|-------------------|---|------------|------------|-------|---------------------|
| | | Time (d) that tumors required to grow from 8 to 12 mm | ATGD‡ | NTGD§ | | |
| 1. Control (inactive oligodeoxynucleotide × 3 only) | 0/10 | 3.9 ± 0.2 | | | | |
| 2. CpG oligodeoxynucleotide 1826 × 3 only | 0/11 | 7.0 ± 0.4 | 3.1 ± 0.4 | | | |
| 3. Single-dose radiation 20 Gy + inactive oligodeoxynucleotide × 3 | 0/10 | 10.8 ± 0.8 | 6.9 ± 0.8 | | | |
| 4. Single-dose radiation 20 Gy + CpG oligodeoxynucleotide 1826 × 3 | 6/10 | 24.2 ± 0.8 | 20.3 ± 0.8 | 17.2 ± 0.8 | 2.49 | |
| 5. Fractionated radiation 2 Gy × 10 + inactive oligodeoxynucleotide × 3 | 0/11 | 5.0 ± 0.3 | 1.1 ± 0.3 | | | |
| 6. Fractionated radiation 2 Gy × 10 + CpG oligodeoxynucleotide 1826 × 3 | 4/10 | 25.0 ± 7.6 | 21.1 ± 7.6 | 18.0 ± 7.6 | 16.36 | |
| 7. Single-dose radiation 20 Gy + inactive oligodeoxynucleotide × 7 | 0/10 | 11.7 ± 2.6 | 7.8 ± 2.6 | | | |
| 8. Single-dose radiation 20 Gy + CpG oligodeoxynucleotide 1826 × 7 | 5/11 | 48.0 ± 7.7 | 44.1 ± 7.7 | 41.0 ± 7.7 | 5.26 | |
| 9. Fractionated radiation 2 Gy × 10 + inactive oligodeoxynucleotide × 7 | 0/11 | 5.3 ± 0.2 | 1.4 ± 0.2 | | | |
| 10. Fractionated radiation 2 Gy × 10 + CpG oligodeoxynucleotide 1826 × 7 | 4/11 | 18.0 ± 5.9 | 14.1 ± 5.9 | 11.0 ± 5.9 | 7.86 | |

*Mice bearing 6 mm tumors in the right hind leg were given s.c. 100 µg CpG oligodeoxynucleotide 1826 or inactive control oligodeoxynucleotide 2138 when tumors were 6 mm in diameter, when tumors grew to 8 mm in diameter, and again 1 week later. When the agents were combined with irradiation, oligodeoxynucleotides were given either thrice, as above, or seven times: when tumors were 6 and 8 mm and once weekly thereafter. Local tumor irradiation was initiated when tumors were 8 mm. A dose of 20 Gy was delivered either in a single dose or in 10 doses of 2 Gy given twice daily for 5 days. In each case, the second oligodeoxynucleotide treatment was given 3 hours after the first dose of radiation.

†Number of mice with permanent tumor regression over total mice in the treatment group. Mice with tumor cures were not included delay analysis.

‡ATGD caused by CpG oligodeoxynucleotide 1826, radiation, or both agents is defined as the time (in days) tumors required to reach 12 mm from the time of treatment at 8 mm minus the time (in days) control tumors required to grow from 8 to 12 mm.

§NTGD is defined as the time (in days) for tumors to reach 12 mm in the mice treated with the combination of CpG oligodeoxynucleotide 1826 and radiation minus the time (in days) to reach 12 mm in mice treated with CpG oligodeoxynucleotide 1826 alone.

||Enhancement factors were obtained by dividing NTGD in mice treated by CpG oligodeoxynucleotide 1826 plus radiation by ATGD in mice treated with radiation only.

Figure 1B shows that there was a difference in the pattern of the growth and regression of cured tumors in the combined treatment groups between those that received single-dose radiation and those that received fractionated irradiation. Four tumors that received fractionated irradiation and seven doses of CpG oligodeoxynucleotide 1826 grew during the first 5 days of radiation treatment and three of the them grew for an additional 5 days afterward, reaching sizes between 9 and 14 mm in diameter before they started to slowly regress. These tumors fully regressed 19 to 32 days after the initiation of radiation treatment. In contrast, tumors cured by CpG oligodeoxynucleotide 1826 (seven doses) plus 20 Gy single-dose radiation grew only slightly for 2 days after radiation but then rapidly regressed between 7 and 11 days after irradiation (Fig. 1B).

Effect of CpG Oligodeoxynucleotide 1826 on Fractionated Radiation-Induced TCD₅₀. To quantify the CpG oligodeoxynucleotide 1826-induced augmentation of tumor curability by fractionated irradiation, TCD₅₀ assays were done. The results are presented in Table 2 and Fig. 2. The percentage of tumors cured increased as the radiation dose increased in both groups. However, to achieve the same percentage of tumor cure, much lower doses of radiation were needed in mice treated with CpG oligodeoxynucleotide 1826 as illustrated by the dramatic displacement of the radiation response curve toward lower radiation doses (left shift) in mice treated with CpG oligodeoxynucleotide 1826 (Fig. 2). CpG oligodeoxynucleotide 1826 reduced the TCD₅₀ value (95% confidence interval) from a total

dose of 83.1 (79.2-90.0) Gy after radiation only to a total dose of only 23.0 (11.5-32.7) Gy. The potentiation of tumor radioresponse at the TCD₅₀ level reached a factor of 3.61, obtained by dividing the TCD₅₀ value of the radiation-alone group with that of the CpG oligodeoxynucleotide 1826 plus radiation group. However, the radiation dose-response curves between the two groups differed in their slope, with the slope of CpG oligodeoxynucleotide 1826 group being shallower. The shallower slope indicates a higher degree of heterogeneity in tumor response to treatment.

Resistance to Tumor Cell Rechallenge of Mice Cured of Fibrosarcoma by Local Tumor Irradiation or CpG Oligodeoxynucleotide 1826 plus Local Tumor Irradiation.

Our earlier report showed that the enhancing effect of CpG oligodeoxynucleotide 1826 on tumor radioresponse was largely immunologic, because most of that effect was abolished by suppressing the immune system of tumor-bearing mice with whole-body irradiation (25). In the study reported here, we tested whether mice cured of their fibrosarcoma tumors by CpG oligodeoxynucleotide 1826 plus local tumor irradiation become resistant to fibrosarcoma cells subsequently inoculated either s.c. or i.v. The results in Fig. 3 show that mice cured of their tumor by either radiation only or combination of CpG oligodeoxynucleotide 1826 plus radiation were resistant to subsequent tumor cell challenge compared with previously untreated non-tumor-bearing mice. In normal mice, 100% tumor take were achieved with all tumor cell doses within the

range of 2.5×10^4 to 1×10^5 . In contrast, similar numbers of tumor cells produced no tumor takes in animals cured by either radiation only or CpG oligodeoxynucleotide 1826 plus radiation. Whereas 2.0×10^5 tumor cells produced 50% tumor take in mice with tumors cured by radiation alone, mice treated with combined CpG oligodeoxynucleotide 1826 plus radiation were totally resistant to tumor growth from cell numbers as high as 8.0×10^5 , the largest tumor cell inoculum used in this experiment. Thus, these animals were >60-fold more resistant to tumor

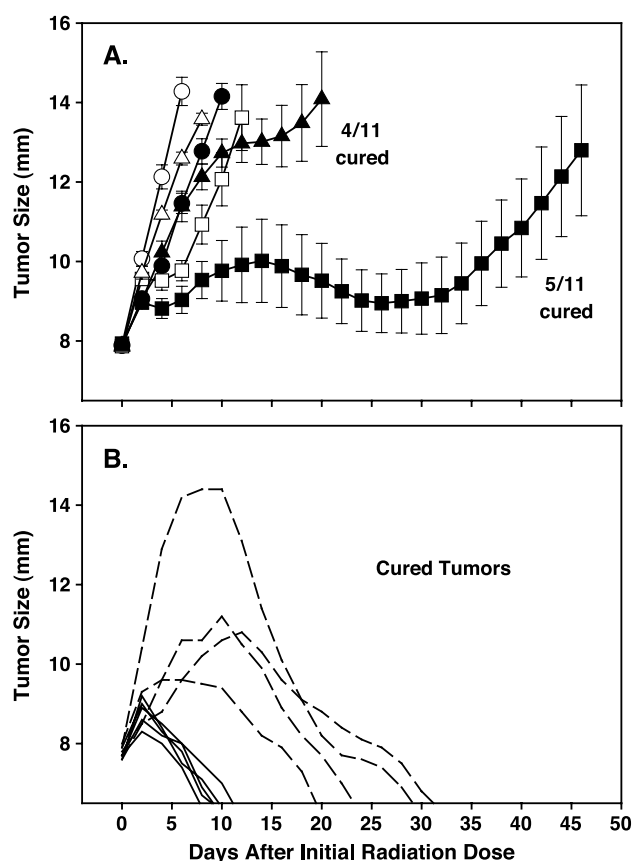


Fig. 1 Effect of CpG oligodeoxynucleotide 1826 on radiation-induced fibrosarcoma tumor growth delay. *A*, mice bearing fibrosarcoma tumors in the leg were treated with CpG oligodeoxynucleotide 1826 (●), its inactive negative control oligodeoxynucleotide 2138 (○), a combination of CpG oligodeoxynucleotide 1826 plus 20 Gy given as single-dose (■) or fractionated radiation (▲), or a combination of inactive oligodeoxynucleotide 2138 plus 20 Gy given as single-dose (□) or fractionated radiation (△). Treatment with CpG oligodeoxynucleotide 1826 and inactive oligodeoxynucleotide 2138 at a dose of 100 μ g per mouse was given s.c. peritumorally once when tumors were 6 and 8 mm and 1 week later. Mice treated with the oligodeoxynucleotides plus radiation received a total of seven injections: at 6 and 8 mm plus five additional treatments at weekly intervals. Points, mean size of 6-11 tumors; bars, SE. Tumors cured by these treatments were excluded from the growth delay analysis. Five of 11 tumors treated with CpG oligodeoxynucleotide 1826 plus single-dose radiation were cured, and 4 of 11 tumors treated with CpG oligodeoxynucleotide 1826 plus fractionated radiation were cured. *B*, individual growth and regression curves for cured mice. Solid lines, CpG oligodeoxynucleotide 1826 plus single-dose radiation; dashed lines, CpG oligodeoxynucleotide 1826 plus fractionated radiation.

Table 2 Control of fibrosarcoma by local irradiation combined with CpG oligodeoxynucleotide 1826: tumors were treated with 10 fractions of irradiation when 8 mm in diameter

| Total radiation dose in Gy* (dose per fraction \times 10) | Proportion of mice cured (%) | |
|--|---|---|
| | Control† (radiation + inactive oligodeoxynucleotide) | CpG oligodeoxynucleotide 1826 + radiation |
| 10 (1.0×10) | | 2/8 (25) |
| 15 (1.5×10) | | 3/8 (38) |
| 20 (2.0×10) | 0/11 (0) | 4/11 (36) |
| 25 (2.5×10) | | 4/7 (57) |
| 30 (3.0×10) | | 5/7 (71) |
| 35 (3.5×10) | | 4/8 (50) |
| 40 (4.0×10) | | 5/8 (63) |
| 45 (4.5×10) | 0/8 (0) | 7/8 (88) |
| 50 (5.0×10) | | 7/8 (88) |
| 55 (5.5×10) | 0/8 (0) | 4/5 (80) |
| 60 (6.0×10) | 0/7 (0) | |
| 65 (6.5×10) | 0/8 (0) | 5/8 (63) |
| 70 (7.0×10) | 0/8 (0) | |
| 75 (7.5×10) | 1/8 (13) | |
| 80 (8.0×10) | 2/8 (25) | |
| 90 (9.0×10) | 7/8 (88) | |
| TCD ₅₀ (95% confidence interval) | 83.1 (79.2-90.0) | 23.0 (11.5-32.7) |

*Sum of 10 equal fractions given twice daily for 5 consecutive days.

†Active CpG oligodeoxynucleotide 1826 or inactive control oligodeoxynucleotide 2138 was given s.c. (100 μ g) for 7 injections beginning when tumors were 6mm in diameter (see Table 1 for details).

cell rechallenge than normal untreated mice, showing $\sim 100\%$ tumor take after rechallenge with 1.25×10^4 tumor cells.

The second experiment assessed the ability of cured mice to resist formation of tumor nodules in the lung after i.v. inoculation of tumor cells. Cured mice used for this assay were obtained from the tumor growth delay (Fig. 1) and tumor cure (Fig. 2) experiments. As shown in Table 3, the number of tumor nodules in the lung of normal mice increased as the number of inoculated tumor cells increased, from a median number of nodules of 14.5 after 2.5×10^4 tumor cells to 106.0 following the injection of 1×10^5 cells. All normal mice developed lung nodules. Mice cured of their tumors were highly resistant to i.v. tumor cell rechallenge as evidenced by both a lower incidence of artificial metastases and the reduction in their absolute number. Like animals rechallenged by the s.c. route, mice cured by CpG oligodeoxynucleotide 1826 plus radiation were much more resistant than those cured by radiation only. To illustrate, only 3 of 9 (33%) mice treated with CpG oligodeoxynucleotide 1826 plus irradiation developed lung nodules with a median number of zero from an inoculum of 1.2×10^6 cells, whereas a 48-fold lower number of i.v. inoculated cells (2.5×10^4) produced tumor nodules in the lung of 6 of 6 (100%) normal mice with a median number of 14.5 nodules.

Effect of CpG Oligodeoxynucleotide 1826 on Radio-response of a Nonimmunogenic Tumor. Our earlier report (25) and the results of the present study showed that CpG oligodeoxynucleotide 1826 dramatically enhanced radioresponse of an immunogenic tumor. Because human tumors are considered to be, in general, weakly immunogenic, it was important to test

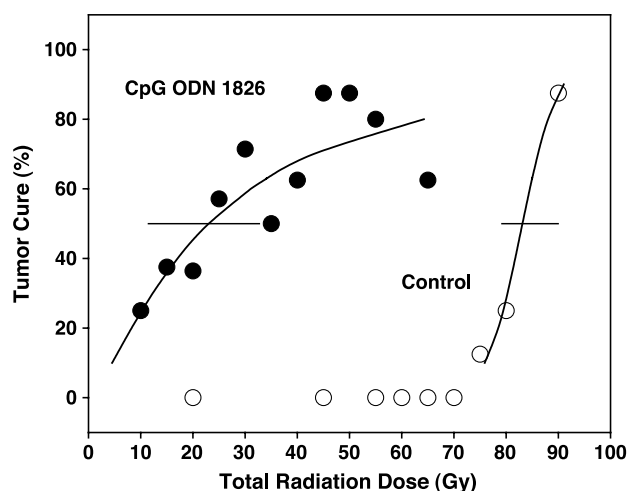


Fig. 2 Effect of CpG oligodeoxynucleotide 1826 on tumor radio-curability. Percentage of tumor cures was plotted as a function of radiation dose. Mice bearing fibrosarcoma tumors in the leg were exposed to a range of fractionated doses when 8 mm in diameter, and they were treated seven times with the active CpG oligodeoxynucleotide 1826 (●) or inactive oligodeoxynucleotide 2138 (○) at a dose of 100 μ g per mouse given s.c. peritumorally when tumor diameters were 6 and 8 mm and once weekly for 5 additional weeks. TCD₅₀ (dose of radiation needed to produce 50% tumor cures in irradiated mice) was determined at 100 days after irradiation. Bars, 95% confidence interval.

whether CpG oligodeoxynucleotide 1826 is also effective against tumors and whether it enhances their radioresponse. We tested this using a syngeneic murine sarcoma, designated nonimmunogenic fibrosarcoma, growing in the leg of mice (27, 28). Nonimmunogenic fibrosarcoma is a more radio-resistant tumor than fibrosarcoma (28). CpG oligodeoxynucleotide 1826 at a dose of 100 μ g was given either s.c. peritumorally, as in the above experiments with fibrosarcoma, or i.t. thrice (once tumors grew to 6 mm, once they measured 8 mm, and again 7 days later). Tumors were irradiated with 30 Gy single-dose radiation when they grew to 8 mm in diameter. In this group, the second dose of CpG oligodeoxynucleotide 1826 was given at 3 hours after irradiation. Treatment with CpG oligodeoxynucleotide 1826 alone significantly delayed the growth of nonimmunogenic fibrosarcoma. Although tumors in untreated mice grew from 6.0 to 12.0 mm in diameter in 9.8 ± 0.5 days, they needed 11.7 ± 0.5 days ($P = 0.026$) and 14.4 ± 1.5 days ($P = 0.028$) to reach the same size in mice treated with CpG oligodeoxynucleotide 1826 s.c. and i.t., respectively. CpG oligodeoxynucleotide 1826 was also strongly effective in augmenting radioresponse of nonimmunogenic fibrosarcoma tumor to radiation (Fig. 4). The tumor growth delay after the combined treatment was more than the sum of tumor growth delays caused by either irradiation or CpG oligodeoxynucleotide 1826, thus indicating the ability of CpG oligodeoxynucleotide 1826 to enhance tumor radioresponse. CpG oligodeoxynucleotide 1826 enhanced tumor radioresponse by a factor of 1.41 and 1.73 when given s.c. and i.t., respectively. Thus, in addition to being effective against the immunogenic

fibrosarcoma, CpG oligodeoxynucleotide 1826 was effective against the nonimmunogenic fibrosarcoma tumor on its own and increased the radioresponsiveness of this tumor.

DISCUSSION

The results of our study showed that treatment of mice bearing large established fibrosarcoma tumors with CpG oligodeoxynucleotide 1826 resulted in remarkably enhanced response of these tumors to fractionated radiotherapy. The effect was shown by a dramatic higher rate of tumor cure than that of tumor radiotherapy alone. The improvement was achieved at all radiation doses used (Fig. 2), the enhancement factor being 3.61 at the TCD₅₀ level. Importantly, this enhancing effect of CpG oligodeoxynucleotide 1826 was observed when combined with daily fractional doses of 2 Gy, a radiation dose fraction commonly used in clinical radiotherapy. The enhancement factor of >3 far exceeds that which we reported previously for single-dose radiotherapy of the same tumor, where the enhancement factor was 1.93 (25). Treatment with CpG oligodeoxynucleotide 1826 was also highly effective in increasing radiation-induced tumor growth delay of tumors not cured by the combined CpG oligodeoxynucleotide 1826 and radiation treatment. Both three and seven doses of CpG oligodeoxynucleotide 1826 were effective. Furthermore, CpG oligodeoxynucleotide 1826 was also effective in increasing tumor radioresponse of a nonimmunogenic fibrosarcoma tumor. Taken together, these observations imply that CpG has potential to be beneficial in clinical radiotherapy.

The rate of tumor cure increased as the dose of radiation increased in mice treated with either radiation alone or both CpG oligodeoxynucleotide 1826 and radiation. However, this radiation dose dependency of tumor cure was less evident in the CpG oligodeoxynucleotide 1826 plus radiation group as evidenced by the shallower slope of the radiation dose-response curve (Fig. 2). The shallow slope is most likely the reflection of heterogeneity

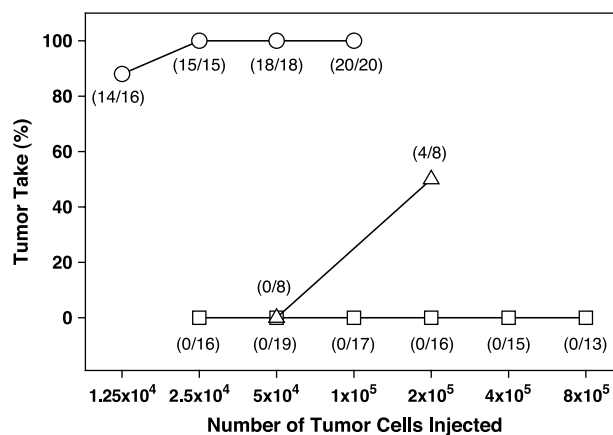


Fig. 3 Resistance of cured mice to reinoculation of tumor cells. Mice cured of their primary tumor after irradiation alone (△) or after treatment with CpG oligodeoxynucleotide 1826 plus irradiation (□) were reinoculated with fibrosarcoma tumor cells 100-120 days after local tumor irradiation. Age-matched untreated mice were used as controls (○). Mice were injected s.c. on the abdomen with graded doses of fibrosarcoma tumor cells and tumor takes observed for up to 2 months after inoculation. Numbers in parentheses, tumor takes over total injection sites.

Table 3 Effect of CpG oligodeoxynucleotide 1826 on lung tumor formation in cured mice rechallenged i.v. with fibrosarcoma cells

| No. tumor cells injected i.v. | Normal | | Radiation + inactive oligodeoxynucleotide | | CpG oligodeoxynucleotide 1826 + radiation | |
|-------------------------------|-----------------------------------|---------------------------------|---|---------------------------------|---|---------------------------------|
| | Proportion of mice with tumor (%) | Median no. lung nodules (range) | Proportion of mice with tumor (%) | Median no. lung nodules (range) | Proportion of mice with tumor (%) | Median no. lung nodules (range) |
| 2.5×10^4 | 6/6 (100) | 14.5 (8-25) | | | | |
| 5.0×10^4 | 7/7 (100) | 42.0 (1-61) | | | | |
| 1.0×10^5 | 6/6 (100) | 106.0 (62-150) | 1/6 (17) | 0.0 (0-23) | 0/9 (0) | 0.0 (0-0) |
| 3.0×10^5 | | | 3/5 (60) | 4.0 (0-101) | 2/9 (22) | 0.0 (0-5) |
| 6.0×10^5 | | | 1/5 (20) | 0.0 (0-185) | 2/9 (22) | 0.0 (0-15) |
| 9.0×10^5 | | | | | 3/9 (33) | 0.0 (0-18) |
| 1.2×10^6 | | | | | 3/9 (33) | 0.0 (0-1) |

NOTE: Cured mice used for this assay were obtained from the tumor growth delay and tumor cure experiments. Normal age-matched mice were used as untreated controls. Groups of mice were injected i.v. with a range of viable fibrosarcoma cells 100-120 days after their primary tumors in the leg were locally irradiated. The number of tumor nodules in the lungs was determined 14 days after i.v. challenge.

of antitumor response of the host mice to CpG oligodeoxynucleotide 1826. Similar heterogeneity in fibrosarcoma radio-curability was observed in our earlier studies that combined treatment with *C. parvum* plus radiotherapy (31, 32). The least heterogeneity was observed in mice whose immune function was immunosuppressed by whole-body irradiation (32). More recently, we showed that whole-body irradiation greatly reduced the ability of CpG oligodeoxynucleotide 1826 to increase tumor radiocurability (25). Taken together, these observations show that although CpG oligodeoxynucleotide 1826 greatly enhanced tumor radiocurability through the engagement of the immune system there was still significant variability in the response the individual mice could mount.

Variability in tumor response to the combined treatment was also manifested in the tumor growth delay treatment end point. Because fibrosarcoma grows rapidly, 20 Gy total dose given in 2 Gy fractions twice daily caused only a small delay in tumor growth, and the effect of CpG oligodeoxynucleotide 1826 on radiation response started to be manifested only several days after initiation of radiation, when tumors had already grown to a considerable size. Some large tumors (Fig. 1B) started to regress after they first grew to 9 to 14 mm in diameter, indicating that once mounted the antitumor response was capable of eliminating many tumor cells. When the radiation dose was large, as was the case with 20 Gy single dose in Fig. 1, tumors that were cured after addition of CpG oligodeoxynucleotide 1826 regressed soon after radiation was delivered (Fig. 1B).

The mice that were cured after the combined CpG oligodeoxynucleotide 1826 plus radiation treatment were highly resistant to rechallenge with tumor cells, inoculated either s.c. or i.v. 100 to 120 days after treatment of the primary tumors. The mice that were cured by radiotherapy only were also resistant to tumor cell rechallenge, but the magnitude of their resistance was lower. These observations suggest that the systemic antitumor rejection response generated by CpG oligodeoxynucleotide 1826 may operate against tumors long after exposure to the agent and might prevent or reduce metastatic development or even cause regression of tumors present during radiotherapy at sites remote from the primary tumor.

In an earlier study (25), we reported that tumors that regressed after treatment with CpG oligodeoxynucleotide 1826 plus radiotherapy were heavily infiltrated with host mononuclear cells, primarily lymphocytes. Although we have not yet

investigated the specific nature of the antitumor immune rejection response, the results of other studies suggest that CpG oligodeoxynucleotide induces an antigen-specific, anti-tumor T-cell response (33-35). Injection of CpG oligodeoxynucleotide creates a Th1-like cytokine/chemokine milieu and lymphadenopathy in the draining lymph nodes (33, 34). Among cells within the enlarging lymph nodes are many dendritic cells that express increased levels of costimulatory molecules and MHC (33). CpG oligodeoxynucleotide activation of dendritic cells promotes strong memory T-cell responses (35). CpG oligodeoxynucleotide-primed mice respond to a subsequent antigen injection in the same anatomic region with a strong Th1-based response and high levels of CTL even several weeks after the CpG oligodeoxynucleotide injection (33, 34). Based on these observations, we hypothesize, that when radiotherapy is given after CpG oligodeoxynucleotide injection, tumor antigens released from dying tumor cells are taken up by activated dendritic cells, leading to the induction of a

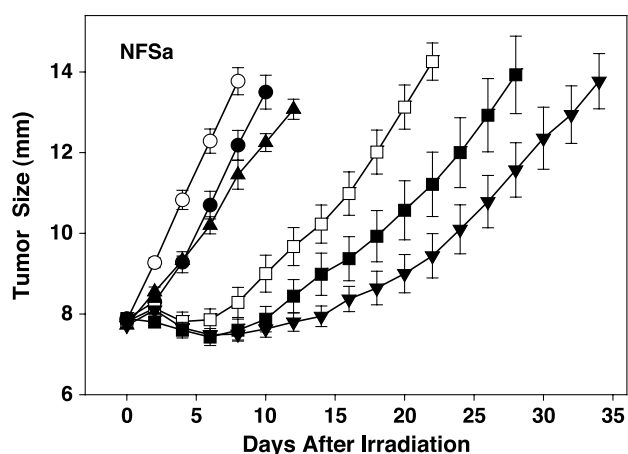


Fig. 4 Effect of CpG oligodeoxynucleotide 1826 on radiation-induced nonimmunogenic fibrosarcoma tumor growth delay. Mice bearing nonimmunogenic fibrosarcoma tumors in the leg were untreated (○) or treated with CpG oligodeoxynucleotide 1826 s.c. peritumorally (●), CpG oligodeoxynucleotide 1826 i.t. (▲), 30 Gy local tumor irradiation (□), or a combination of CpG oligodeoxynucleotide 1826 given s.c. (■) or i.t. (▼) plus 30 Gy irradiation. Treatment with CpG oligodeoxynucleotide 1826 at a dose of 100 μg per mouse was given thrice when tumors were 6 mm, when tumors were 8 mm, and 1 week later. Bars, SE.

tumor-specific T-cell response. Results of the present study support this notion: mice cured of their tumors by the combined CpG oligodeoxynucleotide 1826 plus radiotherapy were more resistant to rechallenge than mice cured by radiotherapy only, and their resistance was long lasting. As noted earlier, the distribution of TLR9 expression differs between humans and mice: B cells and plasmacytoid dendritic cells are positive in both species, but myeloid dendritic cells and monocytes are positive only in mice. Consequently, the cytokine profile induced by CpG oligodeoxynucleotide injection differs between mice and humans (36). Therefore, it is impossible to directly extrapolate across these species to predict that the same synergy between CpG and radiotherapy will occur in humans; clinical trials will be required to confirm this. Nevertheless, in recent human clinical trials, a CpG oligodeoxynucleotide has proven to be a very effective and well-tolerated adjuvant to improve vaccine responses (37–39), indicating that activation of B cells and plasmacytoid dendritic cells is sufficient to promote a strong adaptive immune response in humans, without direct activation of the myeloid dendritic cells or monocytes.

To our knowledge, the present investigation and our previous study (25) are the firsts to focus on the efficacy of CpG oligodeoxynucleotide 1826 plus radiotherapy, although others have reported on combinations of CpG oligodeoxynucleotides with tumor vaccines, antitumor antibodies, chemotherapy, and other immunotherapies (23). CpG oligodeoxynucleotide monotherapy works best in many tumor models when injected i.t. or peritumorally but generally has little activity when injected at a distant site (18–20). CpG oligodeoxynucleotides induce tumor regression in a few models even when given systemically or injected at a distant site (17, 21, 22). Thus, the most efficacious route of CpG oligodeoxynucleotide administration is controlled by host and tumor variables not yet fully defined. Recently, Weigel et al. (24) reported that systemic CpG oligodeoxynucleotides enhance the antitumor effects of the chemotherapeutic agents cyclophosphamide and topotecan and improve survival after surgical resection of murine rhabdomyosarcoma. Although neither cyclophosphamide nor CpG oligodeoxynucleotide alone was curative, the combination resulted in long-term survival of 15% to 40% of mice. Thus, CpG oligodeoxynucleotides can be combined with a variety of therapeutic approaches, including radiotherapy. Current clinical trials with CpG oligodeoxynucleotides showed them to be well tolerated even after weekly dosing for >1 year and to be highly immunostimulatory (40). Our animal tumor models have provided compelling evidence that CpG oligodeoxynucleotide in combination with fractionated radiotherapy is a strong candidate treatment strategy for further clinical development.

The interactions between the immune system and radiation resulting in improvement of tumor control are multiple and complex and have been reviewed recently (41). Tumor radiation may alter tumor immunogenicity, up-regulate expression of inflammatory mediators, induce immunomodulatory cytokines, and initiate both T-cell-dependent and T-cell-independent cascades of antitumor immune responses, all of which are likely to be augmented when radiation is combined with immunomodulating agents. These processes may improve the efficacy of radiotherapy of both immunogenic and nonimmunogenic tumors. Our previous report (25) and the present study have established

that CpG oligodeoxynucleotide 1826 markedly improves tumor response to single-dose and fractionated radiotherapy and that this requires a functional immune system. Further studies will be required to more fully elucidate the mechanisms of these initial observations on the actions and interactions of CpG oligodeoxynucleotides and radiotherapy.

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