Therapeutic Synergism of Gemcitabine and CpG-Oligodeoxynucleotides in an Orthotopic Human Pancreatic Carcinoma Xenograft

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Abstract
CpG-oligodeoxynucleotides (CpG-ODN) exhibit potent immunostimulatory activity by binding with Toll-like receptor 9 (TLR9). Based on the finding that TLR9 is highly expressed and functional in pancreatic tissue, we evaluated the antitumor effects of chemotherapy combined with CpG-ODNs in the orthotopic mouse model of a human pancreatic tumor xenograft. Chemotherapy consisted of the maximum tolerated dose of gemcitabine (i.v., 100 mg/kg, 3qdx4). CpG-ODNs were delivered (i.p., 20 μg/mouse), weekly, after the end of chemotherapy. CpG-ODNs alone had little effect on tumor growth, whereas gemcitabine alone significantly delayed the median time of disease onset (pulpal i.p. tumor) and of bulky disease development (extensive peritoneal tumor burden), but did not enhance survival time. When the gemcitabine regimen was followed by administration of the immunostimulator, development of bulky disease was delayed, survival time was significantly improved (median survival time, 166 days; P < 0.02 versus gemcitabine-treated mice). Autopptic examination showed that tumor spread in the peritoneal cavity was reduced to a greater extent than with gemcitabine alone. All treatment regimens were well-tolerated. The use of nude mice excluded a T cell–mediated immune response, whereas the high pancreatic expression of TLR9 might have contributed to the tumor response. The clear improvement of survival observed in an orthotopic murine model of human pancreatic cancer by the combined use of CpG-ODNs with chemotherapy suggests the promise of this therapeutic regimen in the clinical setting. (Cancer Res 2005; 65(14): 6388-93)

Introduction
Carcinoma of exocrine pancreas is a devastating disease characterized by a very poor prognosis, with most patients dying within 6 months after diagnosis. Surgery is an option in <20% of patients, most of whom develop recurrent disease. Chemotherapy is considered the conventional systemic treatment of advanced pancreatic cancer. Gemcitabine (2’,2’-difluoro-2’-deoxycytidine), a deoxycytidine analogue with broad-spectrum activity against solid tumors, is the best available treatment option (1). Although this drug improves the quality of life in many patients, it prolongs survival of pancreatic cancer patients by only ~1 month (2, 3). Thus, new antitumor therapies for these patients are needed.

One potential strategy for treatment of pancreatic cancer is targeted immunotherapy. The discovery of a series of innate immunospecific receptors activated by pathogen-associated molecular patterns provides new possibilities for a targeted activation of this immunity. Among the innate immune-specific receptors, the best characterized are the Toll-like receptors (TLR; ref. 4). These receptors were initially identified on cells of the immune system, but it has become increasingly clear that they are also expressed on nonprofessional immune cells (5). Interestingly, recent studies of type 1 diabetes reveal evidence of TLR transcript expression by pancreatic islet cells (6, 7).

In the present study, we conducted an in-depth analysis of TLR expression and functionality in murine pancreatic tissue with particular focus on TLR9. This receptor species recognizes specific oligodeoxynucleotide sequences with unmethylated CpG motifs (CpG-ODN) present on bacterial genomic DNA (8). In different experimental tumor models and in a small phase I clinical trial, treatment with synthetic CpG-ODNs alone or in combination with chemotherapy or radiotherapy was shown to exert antitumor activity (9–13). We found that the TLR9 is highly expressed in pancreas, providing an opportunity to evaluate the relative contribution of innate immunity in combating pancreatic carcinoma. Using an orthotopically implanted athymic nude mouse model, we analyzed the antitumor effects of the combination of gemcitabine and CpG-ODNs against a human pancreatic tumor xenograft. Immunotherapy was delivered after the complete chemotherapy regimen because an alternating sequence of the two therapies may result in increased toxicity (10), and immunotherapy is more effective when tumor burden is low (14). Our results indicate significant increase in tumor response of mice receiving both therapies compared to those treated with gemcitabine alone. The expression of TLR9 protein in the murine pancreas may be a critical factor in this effect.

Materials and Methods

Drugs and Synthetic Oligodeoxynucleotides
Gemcitabine (GEMZAR, Eli Lilly Italia S.p.A., Sesto Fiorentino, Italy) was supplied as lyophilized product, which was then dissolved in sterile saline. Purified, single-stranded, phosphorothioated ODN 1826 (5’-TCCAT-GAGCTTCCTGACGT-3’) containing CpG motifs (15) was synthesized under endotoxin-free conditions by Coley-Pharmaceutical Group (Ottawa, Canada). Phosphorothioate modification was used in order to reduce the
susceptibility of the ODN to DNase digestion, thereby significantly prolonging its half-life in vivo. CpG-ODN was dissolved in sterile saline.

**Toll-like Receptor 9 Expression and Functionality in Pancreatic Tissue**

Pancreas harvested from wild-type C57BL/6 mice and leukocytes harvested from wild-type and TLR9−/− (knock-out) C57BL/6 mice were lysed in sample buffer [0.1 mol/L NaCl, 0.01 mol/L Tris-Ci (pH 7.6), 0.001 mol/L EDTA (pH 8.0), 1% Triton X-100, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 mmol/L phenylmethylsulfonyl fluoride] at 4°C for 1 hour, followed by centrifugation. Proteins were quantified by the bicinchoninic acid method (BCA protein assay kit, Pierce, Rockford, IL). Samples were placed in 2× sample buffer [100 mmol/L Tris-HCl (pH 6.8), 200 mmol/L dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol], and boiled at 70°C for 10 minutes, rapidly transferred to ice, resolved on 8% SDS-polyacrylamide gels, and transferred to nitrocellulose membrane (Millipore Corp., Bedford, MA). After blocking with 5% nonfat dry milk, proteins were hybridized for 1 hour with biotinylated anti-mouse TLR9 monoclonal antibody (HBT, clone 5G5, Uden, the Netherlands) diluted 1:25. After incubation with peroxidase-conjugated streptavidin (Vectastain ABC System kit, Vector Labs., Burlingame, CA) for 1 hour, membranes were washed and developed with enhanced chemiluminescence light detection reagents (Amersham Bioscience, Milan, Italy).

For chemokine quantitation, athymic nude mice (three to five animals per group) were injected i.p. with CpG-ODN 1826 (20 μg/mouse) or saline and euthanized 1 hour later, when pancreas and hind muscles were removed, washed twice in saline, and submitted for chemokine extraction using a modification of the PERFEXT method (16). Levels of keratinocyte-derived chemokine, the functional homologue of human IL-8 in mice, were determined by ELISA (R&D Systems, Minneapolis, MN).

**Antitumor Activity Studies**

**Tumor cells.** GER human pancreatic cancer cells (17) were kindly supplied by Dr. A. Scarpa (University of Verona, Italy) and maintained in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS (v/v) Life Technologies Inc., Gaithersburg, MD).

**Mice and anesthesia.** All experiments were carried out using female athymic nude mice (Charles River, Calco, Italy) at 8 to 10 weeks of age (20-26 g body weight). Mice were maintained in laminar flow rooms at constant temperature and humidity, with food and water given ad libitum. Experimental protocols were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan, according to United Kingdom Coordinating Committee on Cancer Research guidelines (18). Mice were anesthetized using a solution of ketamine (Ketavet 50, Merial, United Kingdom Coordinating Committee on Cancer Research guidelines) and xylazine (Rompun, Bayer, Germany) for injections (18). Mice were anesthetized using a solution of ketamine (Ketavet 50, Merial, United Kingdom Coordinating Committee on Cancer Research guidelines) and xylazine (Rompun, Bayer, Germany) for injections (18).

**Western blot analysis of TLR9 expression.** Lanes 1 and 2, leukocyte extracts from TLR9+/+ mouse; lane 3, leukocyte extracts from TLR9−/− mouse; lane 4, leukocyte extracts from TLR9−/− mouse. Bands presented were visualized with a gel imaging system (Syngene, Frederick, MD) and quantified using the Image-G현 software (Syngene, Frederick, MD).

**Experimental model.** GER human pancreatic tumor cells (106 cells in 200 μL/mouse) were orthotopically implanted in anesthetized athymic nude mice as described (19). Briefly, a small left abdominal flank incision was made and the spleen exteriorized. Tumor cells were injected subcapsularly in a region of the pancreas just beneath the spleen. A successful subcapsular intrapancreatic injection of tumor cells was identified by the appearance of a fluid bleb without i.p. leakage. All surgical procedures were done under sterilized conditions with a 10× microscope (SMZ800 Nikon).

At 7, 14, 21, 30, and 48 days after tumor injection, four mice per time point were euthanized, a complete necropsy was done, and tumor size as well as evidence of metastases in the peritoneal cavity were recorded. For histologic procedures, lung, heart, diaphragm, liver, gastrointestinal tract, spleen, kidneys, and genital tract were collected, fixed in 4% formalin, embedded in paraffin, sectioned (4 μm), and stained with H&E. Tumor cells detected within the parenchyma or on the peritoneal surface of abdominal organs were considered metastases.

**Therapy studies.** Six days after tumor cell injection, mice were randomized into four groups (7-10 mice per group): (a) mice receiving gemcitabine (10 mL/kg body weight) four times at a dose of 100 mg/kg each given i.v. at 3-day intervals (q3dx4); (b) mice receiving CpG-ODNs (200 μL/mouse) seven times at a dose of 20 μg per mouse each given i.p. every 7 days (q7dx7); (c) mice receiving CpG-ODNs six times starting 4 days after the last gemcitabine treatment; and (d) control mice receiving sterile saline i.v. and i.p. All mice were weighed biweekly and observed for tumor onset and growth. Tumor diameters were assessed with a Vernier caliper and tumor volume (mm3) was calculated as: \( V = \frac{\pi \times d_1 \times d_2}{6} \times 2 \), where \( d_1 \) and \( d_2 \) represent the shortest and the longest diameter, respectively. Disease onset was recorded as time when a solid i.p. tumor was first palpable. Bulky disease was considered present when tumor burden was extensive and prominent in the mouse abdomen, i.e., when tumor volume was \( >2000 \text{ mm}^3 \).

Treatment efficacy was assessed based on:

- tumor growth and spread: all mice in a group were sacrificed by cervical dislocation a few days after at least half of the group presented bulky disease, i.e., median time was reached. At necropsy, i.p. tumors were collected and weighed, and the volume of ascites was measured. Organs were inspected macroscopically for evidence of metastatic nodules and fixed for histology.
- survival time: mice were sacrificed by cervical dislocation when abrupt body weight loss (25-30%) or evidence of advanced bulky disease was present. The day of sacrifice was considered as day of death.

Any death in treated mice before that of any control mouse was attributed to treatment toxicity.

**Statistical Analysis**

Data from antitumor activity studies were analyzed using the Mann-Whitney rank test (two-sided), whereas data from chemokine quantitation assay were analyzed using the Student’s t test (unpaired and two-tailed). All analyses were done using GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA).

![Figure 1](image-url)

**Figure 1.** A, Western blot analysis of TLR9 expression. Lanes 1 and 2, leukocyte extracts from wild-type mice (15 and 30 μg proteins, respectively); lane 3, leukocyte extracts from TLR9−/− mice (15 μg proteins); lane 4, pancreatic extracts from wild-type mice (15 μg proteins). B, ex vivo keratinocyte-derived chemokine (KC) production in different mouse tissues. Athymic nude mice (three to five per group) were injected i.p. with CpG-ODN (20 μg/mouse) or saline, sacrificed 1 hour later, and pancreas and muscles were isolated. Keratinocyte-derived chemokine levels in tissues were evaluated by ELISA and values normalized per gram of tissue. Data points represent individual mice. \( P < 0.0001 \) in stimulated mice versus saline-treated mice, by Student’s t test.
Results

Toll-like receptor 9 expression and functionality in pancreas. Western blot analysis of pancreatic extracts and of peripheral blood leukocyte extracts obtained from wild-type and TLR9−/− mice used as positive and negative controls, respectively, revealed high-level TLR9 expression in pancreatic extracts (Fig. 1A). The expression was similar to that detected in leukocyte extracts. Previous studies showed that bacterial CpG motifs act as pathogen-associated molecular patterns to stimulate TLR9-positive cells, leading to secretion of large amounts of cytokines and chemokines (13). The functionality of pancreatic TLR9 was defined based on production of keratinocyte-derived chemokine, by specimens obtained from mice treated 1 hour before with CpG-ODNs or saline. Skeletal muscle, which reportedly does not express TLR9 (20), was used as negative control. Significantly increased keratinocyte-derived chemokine levels (P < 0.0001 by Student’s t test in stimulated mice versus saline-treated mice) were found in pancreatic but not in muscle tissues of mice injected i.p. with CpG-ODNs (Fig. 1B). Keratinocyte-derived chemokine levels detected in pancreatic extracts were not due to blood contamination, because no hemoglobin was detected in the samples (data not shown).

Antitumor activity. Orthotopically implanted human pancreatic GER carcinoma cells (10⁶ cells/mouse) led to invasive tumors in 100% of nude athymic mice, with a reproducible pattern of growth; in two separate experiments, palpable disease (disease onset) was present with a median time of 22 and 24 days, respectively, and extensive peritoneal tumor growth (bulky disease) with a median time of 42 days was observed in both experiments. Necropsy of tumor-bearing mice (four mice per time point) at different time points after cell injection revealed no evident tumor at 7 days, whereas at day 14, all mice presented a single mass (~ 4 mm of size) in the head of pancreas, with no invasion of the surrounding organs. One week later (day 21), all mice presented multifocal tumor masses in the head of the pancreas and metastatic lesions in the diaphragm. At day 34, large tumor masses were present in pancreatic tissues of all mice, and tumor growth was evident in all diaphragms and 50% of the livers. Mice sacrificed at day 48 presented diffuse growth of the tumor in the peritoneal cavity, ascites, and frequent liver and spleen metastases.

Antitumor activity of gemcitabine plus CpG-ODNs was evaluated in two separate experiments with the same experimental design, but different end points, i.e., disease onset/progression and survival time, respectively. Figure 2 shows the results of the first experiment in which median times of disease onset and of bulky disease were determined. Compared with the median time of control mice, mice receiving CpG-ODNs alone (q7dx7, from day 6) showed only a marginal delay in disease onset (median time, 27 versus 24 days). By contrast, mice treated with gemcitabine (q3dx4, from day 6) remained without palpable tumor for a longer time (median time, 43 days; P < 0.0005 versus controls). Mice treated with gemcitabine (as above) followed by CpG-ODNs (q7dx6, from day 20) showed a median time of disease onset at days 43 (P < 0.0005 versus control mice).

Control mice developed bulky disease with a median time of 42 days. A nonsignificant delay in median time of bulky disease was observed in CpG-ODN-treated mice (52 days). In contrast, the median time for developing bulky disease was increased by

![Figure 2](https://www.aacrjournals.org/delays/1253338070807892220.png)
gemcitabine treatment to 62 days (P < 0.05 versus control mice). Mice receiving both chemo- and immunotherapy, despite a similar disease onset median time as in mice treated with gemcitabine alone, developed bulky disease 3 weeks later than in the latter group (median time, 81 days; P < 0.005 versus gemcitabine-treated mice).

In both control and treated groups, all mice were sacrificed 3 to 5 days after bulky disease median time was assessed (K days in Fig. 2), and a complete necropsy was done. As shown in Fig. 3, 100% of control mice presented ascites, and tumor invasion in diaphragm, mesentery and gastrointestinal tract, 75% had liver metastases and 50% spleen metastases at day 48. In the group receiving CpG-ODNs, metastatic spread was reduced in diaphragm (43%), liver (43%), and spleen (14%) at day 56. Mice receiving chemotherapy alone and sacrificed at day 64 all had mesenteric invasion, 86% showed gastrointestinal metastases, 70% ascitic fluid, 57% diaphragmatic invasion, 43% liver invasion, and 14% spleen metastases. In the mice receiving both treatment modalities and sacrificed at day 86, only 63% had ascites and 75% diaphragmatic invasion. Moreover, most of these mice were tumor-free in mesentery (63%), gastrointestinal tract (75%), liver (88%), and none had spleen invasion. Histologic analysis of the organs confirmed the macroscopic observations.

In the experiment aimed to assess mice survival time, even the median time for disease onset and bulky disease could be recorded, and the results were totally comparable to those achieved in the first experiment (data not shown).

The effect on survival time of control and treated mice is reported in Fig. 4. Control mice had a median survival time of 71 days, and chemotherapy alone did not significantly increase it (median survival time, 78 days). By contrast, mice receiving weekly treatments of CpG-ODNs after chemotherapy had a median survival time of 106 days (P < 0.02 versus gemcitabine-treated mice). Moreover, at that time, when all gemcitabine-treated mice were already dead, the surviving mice in the combined treatment group seemed active, with no signs of anorexia, cachexia or accumulation of ascites, although pancreatic tumors were present. One mouse in this group died at day 48.

Discussion

Our results show a remarkable degree of therapeutic synergism between gemcitabine and CpG-ODN 1826 in an orthotopic mouse model of human pancreatic tumor. Indeed, a weekly treatment with the immunomodulator significantly increased the survival time of mice that had already received a full regimen of chemotherapy. Gemcitabine alone significantly delayed tumor onset and development, but did not influence mouse survival. In contrast, tumor-bearing mice receiving both therapies showed a clear advantage in terms of delayed tumor growth and reduced peritoneal spreading, as well as increased survival time. Because CpG-ODNs alone did not affect tumor growth, the effect achieved by the sequential use of the two therapies was clearly more than additive and can be considered therapeutic synergism (21).

Orthotopic models in which the tumor is implanted in the tissue from which the primary tumor arises in humans recapitulate the natural history of the clinical disease, including the metastatic pattern (22). Accordingly, the peritoneal organs of mice in our pancreatic tumor model frequently showed tumor invasion. Chemotherapy or immunotherapy alone only partially limited tumor spread in the abdominal organs, whereas mice receiving the combined therapy had tumors that remained localized in the pancreatic area, with essentially no spread to other organs. Thus, the combined therapy not only effectively delayed tumor growth in the implanted organ, but also inhibited metastasis.

Mice treated with the full chemotherapy regimen followed by CpG-ODN treatments never exhibited body weight loss indicative...
of toxicity, although 1 of the 18 mice died at day 48. Indeed, no lethal toxicity was observed in a previous study (10), combining the optimal regimens of other chemotherapeutic drugs (topotecan or doxorubicin) and CpG-ODN in the same sequential schedule used in this study. These findings are consistent with observations in clinical studies, where no severe adverse events have been related to the use of CpG-ODNs in >500 patients studied (23).

Previous studies in animals have shown that CpG-ODN treatment potentiates the effect of other therapies, including radiotherapy (12) and chemotherapy (11), mainly through a T cell–dependent mechanism. Our study, done in athymic mice in which tumor immunogenicity plays no role in the host immune response, might better represent the clinical situation, in light of the generally poor immunogenicity of human tumors (24).

The immunostimulating activity of CpG-ODNs is mediated through the interaction with TLR9, producing an array of cytokines and chemokines in a coordinated manner. The effectiveness of this orchestrated stimulation of the immune system has been shown in many tumors, but with wide diversity in the outcomes (13). For example, in models using immunocompetent mice, the differences have been related mainly to the immunogenicity of the particular tumor type, which affects the susceptibility to various effector cells (14). The expression of TLR9 by malignant B cells is even a tumor characteristic related to treatment response, because CpG-ODNs directly up-regulate the expression of MHC and of costimulatory molecules in these B cell tumors (25, 26). Little attention has been focused on the expression of TLR by the organs on which the tumor is growing, although TLR expression in the surrounding tumor tissues might well play a role in the immune antitumor response. Our data indicate a high-level expression of TLR9 on pancreatic tissues, and the higher production of keratinocyte-derived chemokine induced by CpG-ODNs in pancreas compared with that observed in muscle supports the hypothesis that even nonimmune cells have a role in CpG-ODN-induced cytokine/chemokine release. The detection of transcripts encoding different TLRs in pancreatic islet cells of patients with type 1 diabetes (6, 7) raises the possibility that functional TLR2 and TLR6 in this organ might be related to the tumor-suppressive effects observed in a model of murine orthotopic pancreatic cancer after intratumoral injection of a synthetic lipopeptide that signals through interactions with those receptors (27).

Preclinical studies indicate that systemic or local administration of CpG-ODNs alone can induce tumor growth inhibition and regression in immunocompetent (11, 12) and nude athymic mice (10). In the present study, treatment with CpG-ODNs alone was only marginally effective against GER pancreatic tumor growth and spread, despite the early initiation of treatment (day 6, no evidence of tumor) and the favorable route of injection (i.p.). By contrast, CpG-ODN treatment significantly reduced tumor growth and spread when given after a full regimen of gemcitabine, resulting in a clearly increased survival time. It seems possible that gemcitabine selects/induces a tumor cell population that is particularly sensitive to innate immunity stimulated by CpG-ODNs. A gemcitabine-mediated selection induction of an aggressive tumor cell population compared with the untreated tumor must be considered, because the delayed onset and growth of the tumor after chemotherapy is not reflected in increased mice survival time.

Our study indicates that, in a human pancreatic tumor orthotopically growing in nude mice, although gemcitabine alone delays tumor growth, only repeated treatments with the CpG-ODN immunomodulator after the complete chemotherapy regimen succeeded in increasing mice survival. These findings assume significance in light of the absence, in our experimental system, of a T cell–mediated immune response, which is dependent on tumor immunogenicity, and suggest the value of clinical studies of the effects of gemcitabine combined with CpG-ODNs on pancreatic carcinoma.

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