Abstract

Purpose: Oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN) are potent activators of innate and adaptive immunity. Recognition of CpG-ODN is mediated by Toll-like receptor 9 expressed by immune cells, endothelial and epithelial cells, and fibroblasts. We examined the antitumor effect of CpG-ODN and the role of administration route on human ovarian cancers growing in the peritoneal cavity of nude mice.

Experimental Design: Mice implanted i.p. with human ovarian carcinoma cells were treated i.p., s.c., or i.v. and assessed for survival and tumor-free incidence. Peritoneal washings were analyzed for keratinocyte chemokine production and for functional and phenotypic profiles as indicators of the cell types involved in mediating the antitumor effects.

Results: IGROV-1-bearing mice treated i.p. survived significantly longer than those treated i.v. or s.c. (P = 0.0005), and nearly half of them (8 of 17) were tumor-free by the end of the experiment, a rate never achieved using a variety of chemotherapeutic drugs. High rates of tumor-free mice were observed in three other ovarian tumor xenografts treated i.p. Compared with peritoneal washings of mice treated s.c. or i.v., those from mice treated i.p. showed the highest level of serum and tissue keratinocyte chemokine, the highest number of natural killer cells and neutrophils, and the highest antiproliferative activity in vitro.

Conclusions: The superior antitumor effect obtained by locoregional administration of CpG-ODN in i.p. tumor-bearing mice with a limited adaptive immune response points to the importance of innate effector cells amplification at the site of tumor growth and suggests the promise of i.p. CpG-ODN in clinical trials for ovarian cancer.

Oligodeoxynucleotides containing dinucleotides with unmethylated CpG motifs (CpG-ODN) are strong activators of both innate and adaptive immune systems (1, 2). Recognition of CpG-ODN is mediated by Toll-like receptor 9 (TLR9), a member of the TLR family, which is critically important in detecting microbial pathogens. TLRs, initially identified on cells of the immune system, are also expressed by nonprofessional immune cells such as endothelial cells, fibroblasts, and epithelial cells (3, 4). Both bone marrow and non-bone marrow-derived cells are thought to be involved in the response induced by TLR agonists.

Successes in preclinical studies using CpG-ODN and early indications of its safe use in humans have led to considerable interest in the clinical development of these agents in the treatment of cancer patients (1, 5). Preclinical studies (6–8) have shown superior antitumor effects after intratumor administration of CpG-ODN; thus far, a mechanistic explanation of the route-dependent effects remains to be elucidated. The critical role ascribed to the adaptive immunity in CpG-ODN antitumor effects have led to clinical trials conducted using systemic administrations (9–11). In clinical practice, ovarian cancer is among the few tumor types suitable for intratumor treatment, because its growth is mostly confined to the peritoneal cavity. In such patients, locoregional delivery of therapeutic agents may be a suitable option.

Ovarian cancer is the fifth most frequent cancer, accounting for about 6% of all cancer death in females. Progress in the treatment of the disease has been made in recent years, with the current 5-year survival rate around 45% (12). Cytoreductive surgery and systemic combination chemotherapy with a platinum drug and a taxane represent the standard of care for ovarian cancer patients (13). The role of immunotherapy for ovarian cancer patients has been addressed in an early clinical trial (14), but to our knowledge the efficacy of CpG-ODN against ovarian tumors has not been addressed clinically.

Here, we examined the effects of CpG-ODN on human ovarian cancers in the peritoneal cavity of nude mice and the role of the administration route in determining these effects. The results indicate the superior antitumor activity after i.p. administration compared with other routes of delivery, with increased mouse survival time and tumor-free rates. Analyses of the cellular and immunologic bases of this effect point to the...
Translational Relevance

Oligodeoxynucleotides containing CpG motifs (CpG-ODN) are potent activators of innate and adaptive immunity through Toll-like receptor 9 expressed by immune cells and other normal cells. CpG-ODNs have received attention as a potential approach to cancer therapy and are being investigated in clinical studies. The present study documents an impressive efficacy of CpG-ODN in the locoregional treatment of orthotopically implanted (i.p.) human ovarian carcinomas in mice. Because this model is representative of abdominal spread of human disease, the preclinical studies may have obvious clinical implications. Indeed, the i.p. chemotherapy is recognized to be effective in ovarian carcinoma patients with minimal residual disease after initial debulking. However, such treatment modality may have toxicity-related drawbacks with the use of cytotoxic agents. In contrast, the good tolerability of i.p. administered CpG-ODN emphasizes therapeutic advantages over conventional agents. Thus, this study provides a preclinical basis for novel i.p. approaches in the treatment of ovarian carcinoma.

Materials and Methods

Mice. All experiments were carried out using 8- to 10-week-old female Swiss nude mice (Charles River). Mice were maintained in laminar flow rooms at constant temperature and humidity, with food and water given ad libitum. Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan according to institutional guidelines.

Oligodeoxynucleotides. Purified, phosphorothioated ODN1826 (5′-TCCATGACGTTCCTGACGTT-3′) containing CpG motifs and control ODN2243 lacking a CpG motif were synthesized by Coley-Biemann (KC) production (15). All experiments were carried out using 8- to 10-week-old female Swiss nude mice (Charles River). Mice were maintained in laminar flow rooms at constant temperature and humidity, with food and water given ad libitum. Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan according to institutional guidelines.

Tumors. IGROV-1 (16), SK-OV-3 (17), OVCAR-5 (18), and OvCa432 (19) human ovarian carcinoma cells were used in the study. The IGROV-1 tumor was adapted to grow i.p. and maintained by serial i.p. passage of ascitic cells into healthy mice as described (20). For experiments here, 2.5 × 10^6 ascitic cells in 0.2 mL saline were injected i.p. into mice. For experiments with SK-OV-3, OVCAR-5, and OvCa432 tumors, 10^7 exponentially growing cells from culture, suspended in 0.4 mL of saline, were inoculated i.p. In three of the four tumor systems, hemorrhagic ascites with diffuse peritoneal carcinomatosis develops and the animals eventually die. In OvCa432-bearing mice, only 75% developed peritoneal tumors and no mouse died from tumor burden by 180 days.

Experimental groups (5-7 mice) were inspected daily and weighed three times weekly. Survival was the endpoint of the study and the median day of death (median survival time) was calculated for each group. Antitumor activity was assessed as T/C%, the ratio of median survival time in treated mice with that in control mice. Antitumor activity was assessed as T/C%, the ratio of median survival time in treated mice with that in control mice. "Cures" if tumor-free at necropsy.

Results

CpG-ODN activity against i.p. ovarian tumors and influence of administration route. Mice xenografted i.p. with IGROV-1 human ovarian carcinoma cells were injected i.p., i.v., or s.c. with CpG-ODN delivered q7dx4 at a dose of 20 μg/mouse, corresponding to about 1 mg/kg, starting the day after tumor cell inoculation. Control mice were treated i.p. with saline.
Mice were evaluated for survival and tumor-free rates (Table 1; Fig. 1A). Saline-treated mice developed ascites, with increased abdominal volume and body weight, in a median time of 11 days and died by day 24. Negligible effects on survival were achieved by administering the drug s.c., whereas i.v. delivery of CpG-ODN led to a significantly increased survival time compared with controls (P = 0.0004), with a T/C value of 190%, and 1 LTS of 6 mice. However, the most dramatic effect on survival was observed after i.p. treatment (P = 0.0004 versus controls and P = 0.0005 versus i.v. treated mice) where most mice were LTS. In two replicate experiments, with the i.p. treatment, totally comparable results were obtained (Table 1). Overall, a total of 12 of 17 mice were LTS at the end of the experiment (120-130 days), and necropsy revealed a few small solid tumors in 4 mice, whereas 8 were tumor-free. Treatment using ODN2243 lacking a CpG motif did not increase mouse survival (T/C value of 112%). Analysis of the effect of i.p. CpG-ODN in mice with advanced IGROV-1 tumor burden, with treatment starting 8 days after cell injection, revealed a significant increase in lifespan (T/C value of 162%; P = 0.0044 versus control mice), but all mice died with ascites by day 60 (Table 1; Fig. 1A).

The antitumor efficacy of CpG-ODN delivered i.p. q7d was also assayed in mice bearing the human ovarian tumor cells SK-OV-3, OVCAR-5, and OvCa432. Treatment was continued for 7 weeks, because such tumors grew slower than IGROV-1 tumor. In mice injected with SK-OV-3 cells, a slow-growing tumor developed with ascites onset in a median time of 40 days. CpG-ODN treatment starting 1 day after tumor cell inoculation delayed the onset of ascites and significantly prolonged survival (P = 0.0001), with 5 LTS of 6 mice at day 185. A strong antitumor effect was also observed when treatment started 8 days after cell injection, with 3 of 5 mice alive at the end of the experiment (day 185). In total, 8 of 11 treated mice were LTS, and at necropsy, 7 were tumor-free (Table 1; Fig. 1B). OVCAR-5-bearing mice developed ascites in a median time of 17 days. CpG-ODN treatment starting the day after cell inoculation led to significantly increased lifespan (T/C value of 306%; P = 0.0001). Four of 7 mice died from tumor, with solid nodules and low or no ascites, whereas the 3 LTS at day 180 were tumor-free at necropsy (Table 1; Fig. 1C). The OvCa432 tumor grew very slowly and only 75% of control mice developed i.p. tumors by 180 days after inoculation; at that time, none of the mice treated with CpG-ODN starting the day after cell injection revealed evidence of tumor (data not shown).

CpG-ODN delivered by all routes was well tolerated, although brief diarrheic events were often observed 2 to 4 h after i.p. or i.v. administration. At necropsy, tumor-free mice showed no sign of peritoneal damage.

**Immunologic responses induced by CpG-ODN administered by different routes.** To explore the mechanisms underlying the significant differences in antitumor activity observed among the three routes of CpG-ODN administration, several immunologic variables were evaluated at both systemic (blood and spleen) and local (peritoneal washings and peritoneal tissue) levels in mice treated with CpG-ODN by the different routes at the same dose (20 µg/mouse) used for the antitumor efficacy studies.

**KC levels.** Murine KC is the functional counterpart of human interleukin-8, a chemokine that induces a cascade of events leading to the attraction and accumulation of activated polymorphonuclear neutrophils. In healthy nude mice, ELISA analysis of serum from blood collected 90 min after CpG-ODN treatment by the three different routes revealed higher KC levels in all three groups compared with those in untreated mice, in which KC was barely detectable. The highest KC levels were found in mice treated i.p., in which levels were 2-fold greater than those in i.v. or s.c. treated mice (P = 0.008 versus i.v. treated mice and P = 0.006 versus s.c. treated mice; Fig. 2A). Even in tumor-bearing mice, the i.p. injection of CpG-ODN induced a 2-fold greater KC levels ± SD than those observed in i.v or s.c. treated mice (4,322 ± 962, 1,919 ± 1,337, and 2,041 ± 823 pg/mL, respectively).

Because KC is produced by immune and nonimmune cells in response to TLR9 activation (22), we have also carried out ELISA analysis using peritoneal tissue extracts. Again, the highest level of KC was detected in mice treated by the i.p. route; although the value differed significantly compared with that of control mice (P = 0.0098), it was not significantly higher than those in i.v. or s.c. treated mice (Fig. 2B). The KC detected

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**Table 1.** Antitumor activity of CpG-ODN1826 (20 µg/mouse, every seventh day) on human ovarian tumors xenografted i.p.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Day of first treatment</th>
<th>No. treatment</th>
<th>Route</th>
<th>T/C%*</th>
<th>P †</th>
<th>LTS 1 (d)</th>
<th>Cured mice 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGROV-1</td>
<td>+1</td>
<td>4</td>
<td>i.p.</td>
<td>N/A</td>
<td>0.0004</td>
<td>4/6 (120)</td>
<td>2/6</td>
</tr>
<tr>
<td>SK-OV-3</td>
<td>+8</td>
<td>7</td>
<td>i.p.</td>
<td>N/A</td>
<td>0.0007</td>
<td>4/6 (120)</td>
<td>3/6</td>
</tr>
<tr>
<td>OVCAR-5</td>
<td>+1</td>
<td>7</td>
<td>i.p.</td>
<td>N/A</td>
<td>0.0014</td>
<td>4/5 (130)</td>
<td>3/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>i.v.</td>
<td>190</td>
<td>0.0004</td>
<td>1/6 (120)</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>s.c.</td>
<td>115</td>
<td>NS</td>
<td>0/6</td>
<td>—</td>
</tr>
</tbody>
</table>

**NOTE:** On day 0, tumor cells were injected i.p. in female nude mice.

*Ratio of the median survival time in treated over control mice × 100. Median survival times were 21, 18, and 16 d in controls of the three experiments with IGROV-1 tumor; median survival times were 53 and 34 d in controls of SK-OV-3 and OVCAR-5 tumors, respectively. NA, not assessable, because the majority of mice did not die for tumor burden.

† By two-sided log-rank test over control mice.

LTS: mice alive, with or without tumor, on the day of experiment end (in parentheses).

Number of tumor-free mice/total number of mice at necropsy.
in peritoneal tissue extracts was not due to blood contamination, because no hemoglobin was detected in these samples (data not shown).

**Spleen cell cytotoxic activity.** Spleen cells from mice (4 mice per group) treated with CpG-ODN by the three routes and recovered at 3, 24, and 72 h after treatment were tested for cytotoxic activity in a standard 4-h $^{51}$Cr release assay using IGROV-1 as targets. At all times evaluated, no significant cytolitic activity was observed (<4% at effector-to-target ratio of 50:1; data not shown).

**Functional and phenotypic profile of peritoneal cells.** Peritoneal cells obtained from mice 72 h after CpG-ODN treatment by the three routes were evaluated for cell number (9 mice per group), surface phenotype (3 mice /group), and cytotoxic (3 mice per group) and antiproliferative activity (3 mice per group). Cell counting in peritoneal washings indicated a significantly higher cell number in i.p. treated mice compared with controls ($P = 0.0027$), whereas cell numbers in mice treated i.v or s.c were similar to those in control mice (Fig. 3). Fluorescence-activated cell sorting analysis of the peritoneal cells using direct staining with antibodies PE-F480, PE-NK1.1, or PE-GR-1 to detect markers of macrophages, NK cells, or granulocytes, respectively, revealed a marked increase in the percentage of both NK cells ($P = 0.0056$ versus control mice) and granulocytes ($P = 0.0127$ versus control mice) in mice treated i.p. with CpG-ODN, whereas mice treated s.c. or i.v. exhibited only a slight increase in the percentage of cells positive for the granulocyte marker ($P = 0.0366$ s.c. treated mice and $P = 0.0266$ i.v. treated mice versus control mice; Fig. 4A-D).

**Fig. 1.** Kaplan-Meier plot of the percentage of survivors over time among mice injected i.p. with IGROV-1 (A), SKOV-3 (B), or OVCAR-5 (C) ovarian carcinoma cells. Mice were treated with CpG-ODN1826 (20 µg/mouse at 7-d intervals) starting 1d (open symbols) or 8 d (filled symbols) after cell injection: △, i.p. treated mice; ▽, i.v. treated mice; □, s.c. treated mice; ○, saline-treated mice. Experimental groups consisted of 5 to 7 mice.

**Fig. 2.** KC production induced by injection of CpG-ODN (20 µg/mouse) in serum (A) and peritoneal tissue extracts (B) at 90 min after treatment. KC levels evaluated by ELISA were normalized per gram of tissue. Mean ± SD of KC levels of 3 mice per group. *, $P < 0.01$ versus mice treated i.v. or s.c.; **, $P < 0.01$ versus control mice (Student’s t test).
Peritoneal cells showed no significant cytotoxic activity against IGROV-1 target cells in a standard 4-h $^{51}$Cr release assay (<4% at effector-to-target ratio of 50:1; data not shown). On the other hand, by $[^{3}H]$thymidine incorporation assay, the antiproliferative activity against IGROV-1 cells by total peritoneal cells derived from i.p., i.v., or s.c. treated mice revealed 32%, 24%, and 2% proliferation inhibition; similar analysis using adherence-purified mononuclear macrophages revealed 25%, 16%, and 2% inhibition, respectively.

### Discussion

The present study shows the powerful antitumor effect of CpG-ODN delivered i.p. against human ovarian tumor xenografts growing i.p. in nude mice and shows that this effect is highly dependent on the administration route. Indeed, against IGROV-1 tumor CpG-ODN had less effect when administered i.v. and essentially no effect when delivered s.c., in sharp contrast with the significantly increased survival time after i.p. administration, with most mice still alive at the end of experiments and nearly half of them (8 of 17) tumor-free at necropsy. The dramatic effect of CpG-ODN delivered i.p. was observed regardless of the ovarian tumor model used, because in mice implanted with four different tumors most of treated mice resulted “cure” at experiments end (4-6 months after tumor cell implant), thus indicating that the antitumor effect was not cell specific. Such findings, observed when CpG-ODN treatment started 1 day after tumor cell inoculum, raised the possibility that the CpG-ODN induced immunologic effects that impair cancer cell adhesion and migration. However, in the slow-growing SK-OV-3 tumor, a marked increase in mouse survival was observed even when treatment started 8 days later, thus indicating the ability of i.p. CpG-ODN to affect the growth of already established tumors.

We reported previously the antitumor effect of cytotoxic drugs delivered i.p. according to optimal schedules against the i.p. IGROV-1 tumor xenograft (20). In that study, which included drugs of different mechanistic classes and in clinical use, all of the drugs increased mouse survival and some of them, especially topotecan, achieved a certain number of LTS. However, none of the treated mice was tumor-free at necropsy. Our present findings of “cure” in a sizable proportion of ovarian tumor-bearing mice after i.p. CpG-ODN treatment raise the possibility that this immunomodulating reagent might be superior to current chemotherapies for ovarian cancer.

The i.p. administration of antineoplastic agents has been investigated in clinical studies of peritoneal tumors for more than 40 years (23) based on the rationale that this route of delivery increases exposure of cancer cells to the drug while minimizing systemic toxic effects (24). Clinical advantages of i.p. chemotherapy have been reported in ovarian carcinoma patients with minimal residual disease after debulking (25). Such treatment modality has not received a general acceptance because of technical problems, risk for poor (drug-dependent) tolerance, and reduced quality of life during treatment. However, the clinical advantage of i.p. therapy in selected patients supports the interest of this approach as an effective treatment option. In contrast to chemotherapy, i.p. administration of immunostimulating agents, such as IFN-α, showed good tolerability in ovarian cancer patients, although no conclusions about efficacy could be drawn in that study (14).

In our present preclinical model, locoregional administration of CpG-ODN was not only well tolerated by the mice but also remarkably efficacious. In past and ongoing clinical trials, CpG-ODN is administered s.c. because this route has been reported to effectively activate adaptive immunity (1). However, the immunomodulating activities of CpG-ODN are not restricted to the instruction and reprogramming of adaptive immunity but may also involve amplification of the innate effector cell response. Unlike cells of the adaptive immune response, which can reach the antigen wherever they are activated, innate effector cells gather where the antigen induces their activation. The strong antitumor effect observed in our models only after CpG-ODN i.p. treatment in athymic mice points to the importance of amplification of the innate effector cell response at the site of tumor growth. Consistent with this notion, mice treated by the i.p. route showed a strong increase in peritoneal cell number, especially in granulocytes neutrophils and NK cells, and produced the highest levels of serum and peritoneal tissue KC, which plays a pivotal role in the recruitment and activation of neutrophils and monocytes (26–28). Other cytokines, such as IFN-γ and tumor necrosis factor-α, were not differently modulated by CpG-ODN delivered accordingly the three routes.

Whereas in vitro cytotoxicity assays using spleen cells or cells from peritoneal washing as effector cells and tumor cells as targets revealed no significant cytotoxic activity, total peritoneal cells, containing NK cells, neutrophils, and mononuclear macrophages, inhibited the in vitro proliferation of IGROV-1 cells as did adherence-purified macrophages to a lesser extent. Although interspecies NK reactivity is lower than intraspecies (29), alltogether these results suggest that NK cells might not play a prominent role in the CpG-induced antitumor activity in ascitic ovarian carcinomas xenograft, whereas multiple and presumably synergistic antineoplastic cell populations are induced by CpG-ODN to mediate its antitumor effect. Indeed, a critical role for phagocytes (macrophages and granulocytes) has been reported in the antitumor effects induced by CpG-ODN without T cells in murine tumors (30). Although the antitumor activity of NK cells and macrophages is well...
known, insights into the potential role of neutrophils in anticancer therapy have only recently begun to emerge (31–38), because these cells have been long recognized only as key players in innate defenses against microbial infections. Neutrophils express TLR9 mRNA and protein (39–41), but little is known about the effect of TLR9 signaling on neutrophil functions (42).

It is likely that KC is the main cytokine involved in the recruitment and activation of innate immune effector cells at tumor site and that TLR9-expressing nonprofessional immune cells, such epithelial and endothelial cells, play a prominent role in the cytokine production. Accordingly, high levels of serum KC were observed after i.p. administration of CpG-ODN in chimeric C57BL/6 mice reconstituted with TLR9−/− bone marrow-derived cells and therefore expressing TLR9 exclusively in nonhematopoietic cells.4 Thus, the superior antitumor effect observed after i.p. CpG-ODN injection is probably related with the contribution of local nonprofessional immune cells in KC production.

The powerful antitumor effect of the TLR9 agonist CpG-ODN delivered locoregionally in a panel of ascitic models of human ovarian carcinoma resulted in a tumor-free status for a sizable proportion (about 50%) of mice with a genetically limited adaptive immune response. Such an effect, superior to those obtained by different established cytotoxic drugs, provides evidence of the importance of innate effector cells in the antitumor activity induced by the CpG-ODN immunomodulating agent. Clinical trials of i.p. CpG-ODN treatment in ovarian carcinoma patients with minimal residual disease might now be contemplated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Eradication of Ovarian Tumor Xenografts by Locoregional Administration of Targeted Immunotherapy

Michelangela De Cesare, Claudia Calcaterra, Graziella Pratesi, et al.


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