Implication of Macrophages in Tumor Rejection Induced by CpG-oligodeoxynucleotides Without Antigen

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ABSTRACT

Phosphorothioate oligodeoxynucleotides containing CpG motifs (CpG-ODNs) display broad immunostimulating activity and have potential applications in cancer immunotherapy. To investigate the antitumor activity of CpG-ODNs and to study the role of macrophages and lymphocytes in tumor rejection, CpG-ODN’s effects on 9 L glioma cells were assessed in Fisher rats, depleted or not in macrophages, in nude mice, and in SCID mice. In nondepleted rats, intratumoral injections with 100 μg of CpG-ODNs on days 5, 12, and 19, after s.c. 9 L cell inoculations, resulted in an 84% reduction of the tumor volumes, when compared with controls injected with saline (P < 0.0001). Whereas all control animals developed tumors, more than one-third of the treated rats remained tumor free. Rejection of established glioma induced a specific long-term immunity, as cured rats were protected against a subsequent 9 L injection, but not a RG2 cell inoculation, another syngenic glioma in Fischer rats. Macrophages played a critical role in the early phase of tumor rejection, because the CpG-ODN’s effects were significantly decreased in the rats depleted in macrophages, and none of the macrophage-depleted rats treated with CpG-ODNs rejected the tumor. On the contrary, both nude and SCID mice, which have normal innate immunity, showed a significant decrease of tumor volume when treated with CpG-ODNs when compared with controls. T cells were however involved in a later phase of the tumor rejection, as all nude mice eventually developed tumors despite the initial tumor growth inhibition.

Altogether, these data suggest that immunostimulatory CpG-ODNs induced tumor rejections through an early activation of innate immunity and priming of a specific immune response against glioma cells.

INTRODUCTION

Malignant glioma is the most common primary brain tumor in adults (1). Despite treatment combining surgery, radiotherapy, and chemotherapy, the overall survival is poor, underlying the need for new therapeutic options.

Synthetic ODNs containing CpG motifs flanked by two 5’ purines and two 3’ pyrimidines display strong immunostimulatory activity and promote differentiation of naïve T-helper cells toward a T-helper1 phenotype (2–5). When combined with antigens, CpG-ODNs have been successfully used as adjuvant for immunization in experimental models of bacterial and viral infections (6, 7) or cancer (8, 9). However, the selection and purification of a tumoral antigen is a limiting step in cancer immunotherapy. Recently, we hypothesized that the tumor itself can be used as the source of antigen by direct injections of CpG-ODN within the tumors. We showed previously that intratumoral injections of CpG-ODNs alone induce rejection of established tumors and confer long-term protection against a subsequent tumor inoculation (10, 11).

Antigen-presenting cells, among which macrophages, and B lymphocytes are known to be the primary targets of CpG-ODNs (4, 12), but the respective role of each type of cells in tumor rejection is not known. As human gliomas are regularly infiltrated by macrophages but not B cells (13), the evidence that macrophages mediate CpG-ODNs antitumor effects would hold promise for future clinical trials. To assess the type of immunity involved in tumor rejection, we therefore investigated the efficacy of CpG-ODNs in rats depleted or not in macrophages and in nude and SCID mice.

MATERIALS AND METHODS

ODNs. Purified single-stranded phosphorothioate ODNs were purchased from Eurogentec (Seraing, Belgium). The sequences used in this study were CpG-ODN,-5’TGACTGT-GAACGTTCGAGATGA- in which both CpG motifs have been mutated, as described elsewhere (11). ODN containing a cytosine-guanosine motif; NK, natural killer.

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bresle, France), SCID C57Bl6 mice, or nude mice (CERJ, Lyon, France) were anesthetized with Ketamine (Rhone Mérieux, Lyon, France) and Xylasine (Centrevet, Planoat, France).

For tumor implantation, animals were inoculated s.c. with 10^4 or 10^5 cells resuspended in 100 μl of saline into the right flank. On the indicated days, animals were injected either with 100 μl of isotonic sodium chloride (controls) or CpG-ODNs dissolved in 100 μl of saline in the tumor bed. Tumor volumes were assessed with a caliper every 4 days using the formula: \( \text{π/6 × length × width}^2 \) (11).

Depletion of Macrophages in Vivo. To study the role of macrophages in tumor rejection, a Silica suspension in water (300 mg/ml) was injected i.p. at the dose of 100 mg/rat, 20 and 10 days before, on the same day, and 10 days after tumor inoculation. This regimen was reported to efficiently inactivate macrophages in vivo in Fischer rats (14).

Statistical Analysis. Differences in tumor size among the various groups were determined by the ANOVA repeated-measures test.

RESULTS

Inhibition of Tumor Growth After Injection of Cpg-ODNs into S.c. 9 L Glioma. Rats that had been implanted s.c. with 9 L cells were injected 5, 12, and 19 days later into the tumor bed with either 100 μg of CpG-ODNs, 100 μg of IMM-ODNs, or 100 μg of sodium chloride. By day 23 after the tumor graft, animals treated with CpG-ODNs had tumor volumes that were 84% smaller than controls injected with saline (mean tumor volumes ± SE: 161 ± 92 mm^3 versus 1022 ± 59 mm^3, n = 7/groups, \( P < 0.0001 \)). Whereas all control animals developed tumors, three rats in the CpG-ODN-treated group (43%) remained tumor free over a 3-month period of observation. The antitumor effect of CpG-ODNs was dependent on the CpG motifs, as no significant tumor growth inhibition was seen with IMM-ODN (volumes: 674 ± 5 mm^3, n = 7, \( P = n.s. \)), and all rats developed tumors (Fig. 1).

Similar results were seen in rats implanted s.c. with 10^5 9 L cells and subsequently treated on days 2, 5, and 9 with 50 μg of CpG-ODNs or 100 μl of sodium chloride (mean tumor volumes on day 14 ± SE: 202 ± 15 mm^3 versus 0 ± 0 mm^3, n = 4, \( P = 0.0001 \)), and half of the CpG-ODN-treated rats did not develop any tumors.

Rejection of 9 L Tumors After Second Challenge. To determine whether rejection of s.c. implanted 9 L tumor cells led to a specific long-term immunity, four rats that had rejected 9 L cells after treatment with CpG-ODNs were inoculated 3 months later with 10^6 9 L glioma cells into the left and 10^6 RG2 glioma cells into the right flank. Whereas all animals developed RG2 tumors, all 9 L tumors were rejected.

CpG-ODN Effects in Nude and SCID Mice. Nude mice, which lack T cells, were inoculated s.c. with 10^5 9 L cells and treated on days 2, 5, and 9 with either 50 μg of CpG-ODNs (n = 8) or saline (n = 8). On day 14, animals treated with CpG-ODNs showed a 60% decrease of tumor volume when compared with controls (120 ± 42 mm^3 versus 302 ± 92 mm^3, \( P = 0.03 \) versus Fig. 2). However, all animals eventually developed tumors.

SCID mice, which lack both T and B lymphocytes, were inoculated s.c. with 10^5 9 L cells and treated on days 2, 5, and 9 with either 50 μg of CpG-ODNs (n = 8) or saline (n = 7). Tumors grew poorly in SCID mice, as only four of seven mice developed measurable tumors in the control group. However, in the CpG-ODN-treated group, only one mouse developed a tumor, and the overall growth inhibition reached statistical significance (mean tumor volumes ± SE: 9 ± 9 mm^3 versus 49 ± 19 mm^3, \( P = 0.03 \) versus Fig. 3).

CpG-ODN Effects in Macrophage-depleted Rats. To specifically study the role of macrophages, rats were injected i.p. with either saline (n = 10) or silica (n = 10). On day 0, 10^5 9 L cells were injected s.c., and each group was divided into two groups of five animals each for subsequent intratumoral injections with 50 μg of CpG-ODNs or saline on days 2, 5, and 9 (Fig. 4).

All animals, which were not treated with CpG-ODNs, developed tumors, and silica treatment did not significantly affect tumor growth (mean tumor volumes on day 14 ± SE: 140 ± 32 mm^3 for controls versus 109 ± 45 mm^3 for silica-treated rats, \( P = 0.78 \)).
Treatment of Gliomas by CpG-oligodeoxynucleotides

In agreement with the first set of experiments, CpG-ODNs in nonsilica-treated rats induced a 94% decrease of tumor volume when compared with controls (on day 14: 8 ± 6 mm³ versus 140 ± 32 mm³, P = 0.0001), and two of the five rats did not develop any tumor over a 3-month period of observation.

The CpG-ODN’s effects were significantly decreased in the rats injected with silica, as none of the macrophage-depleted rats treated did reject the tumor. When measured on day 14, animals treated with CpG-ODNs and depleted in macrophages had tumor volumes that were five times those of nonmacrophage-depleted rats (40 ± 12 mm³, versus 8 ± 6, P = 0.02). This effect was even more clear on day 9, with a mean tumor volume higher than in nondepleted rats (22 ± 8 mm³, versus 1 ± 1, P < 0.03) and similar to that of control rats (30 ± 12 mm³).

DISCUSSION

In animal models of cancer, successful immunizations against tumoral antigens with CpG-ODNs as adjuvant have been reported (8, 9). However, this approach is limited by the potential selection of antigen-negative cells, which might allow tumors to escape the immune response, and because relevant tumoral antigens are rarely identified. We here showed that intratumoral injections of CpG-ODNs can induce glioma rejection, without the need for preselection and purification of any tumoral antigen. CpG motifs within the ODN were critical to trigger the immune response, for an ODN without such motifs was inefficient. CpG-ODN’s effects are potent, as >40% of the rats rejected the tumor graft, and an 84% inhibition of tumor growth was observed. These data are in agreement with previous studies, reporting the antitumor activity of CpG-ODNs (10, 11) or bacterial DNA (3) in other murine models. The mechanisms underlying such tumor rejection are unclear but should theoretically at least involve macrophages and/or B cells, which are known to be the primary mediators of CpG-ODNs (4, 12).

Interestingly, macrophages, but not B or T cells, played a critical role in the early phase of tumor rejection. Over the first 2 weeks, a significant tumor growth inhibition was seen in nude mice, which lack T cells, and experiments on SCID mice, which lack both B and T cells, showed similar results. In contrast, inactivation of macrophages significantly reduced the CpG-ODN’s effects, and none of the rats depleted in macrophages rejected the tumor grafts, despite CpG-ODN treatment. CpG-ODN effects were not completely abrogated by silica treatment, either because macrophage inactivation was incomplete or because of cutaneous dendritic cells, whose functions are not affected by silica application (15). CpG-ODNs are known to directly activate macrophages and dendritic cells, which in turn stimulate NK cells through interleukin-12 secretion (4, 12). We have reported previously that neuroblastoma rejection by CpG-ODN in mice critically depends on NK-cell activation (11). Similarly, intratumoral infiltrates with macrophages and NK cells were seen in a fibrosarcoma murine model, after intratumoral injections with DNA extracts of Mycobacter Bovis, which contain CpG motifs (16). Therefore, primary activation of macrophages, which in turn activate NK cells, probably represents an important component in the early phase of tumor rejection.

Besides the innate immunity, several facts suggest that T cells are, however, involved in a later phase: (a) nude mice, which only lack T cells, eventually developed tumors, despite the initial tumor growth inhibition; and (b) CpG-ODN-cured animals were protected against subsequent inoculations with 9 L tumor cells. This immunity was highly restricted to 9 L glioma cells, as cured rats were not protected against RG2, another syngenic glioma in Fischer rats. This long-term, cell type-specific immunity strongly suggests the involvement of memory T lymphocytes. Direct stimulation of T cells by CpG-ODNs has not been clearly demonstrated in the literature, and it is likely that CpG-ODNs favored this specific immunization through stimulation of macrophages and antigen-presenting cells (4, 5). In that respect, it is noteworthy that none of the rats depleted in macrophages rejected the tumor grafts, despite CpG-ODN treatment.

In conclusion, CpG-ODN can induce tumor rejection when injected alone in the vicinity of 9 L glioma. Macrophages play a critical role in mediating the CpG-ODNs effects, a fact that...
suggests that human gliomas, which are infiltrated with macrophages (13), can also benefit from such immunotherapy. By stimulating innate immunity and triggering a specific immune response, CpG-ODNs display a unique antitumor activity overcoming the need for identification of tumoral antigens.

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