Microenvironment and Immunology

TLR9 Agonists Oppositely Modulate DNA Repair Genes in Tumor versus Immune Cells and Enhance Chemotherapy Effects

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Abstract

Synthetic oligodeoxynucleotides expressing CpG motifs (CpG-ODN) are a Toll-like receptor 9 (TLR9) agonist that can enhance the antitumor activity of DNA-damaging chemotherapy and radiation therapy in preclinical mouse models. We hypothesized that the success of these combinations is related to the ability of CpG-ODN to modulate genes involved in DNA repair. We conducted an in silico analysis of genes implicated in DNA repair in data sets obtained from murine colon carcinoma cells in mice injected intratumorally with CpG-ODN and from splenocytes in mice treated intraperitoneally with CpG-ODN. CpG-ODN treatment caused downregulation of DNA repair genes in tumors. Microarray analyses of human IGROV-1 ovarian carcinoma xenografts in mice treated intraperitoneally with CpG-ODN confirmed in silico findings. When combined with the DNA-damaging drug cisplatin, CpG-ODN significantly increased the life span of mice compared with individual treatments. In contrast, CpG-ODN led to an upregulation of genes involved in DNA repair in immune cells. Cisplatin-treated patients with ovarian carcinoma as well as anthracycline-treated patients with breast cancer who are classified as "CpG-like" for the level of expression of CpG-ODN modulated DNA repair genes have a better outcome than patients classified as "CpG-untreated-like," indicating the relevance of these genes in the tumor cell response to DNA-damaging drugs. Taken together, the findings provide evidence that the tumor microenvironment can sensitize cancer cells to DNA-damaging chemotherapy, thereby expanding the benefits of CpG-ODN therapy beyond induction of a strong immune response. Cancer Res; 71(20); 6382–90. ©2011 AACR.

Introduction

The mammalian innate immune system identifies the presence of infection through recognition of pathogen-associated molecular patterns expressed by a diverse group of infectious microorganisms. Various pattern recognition receptors are involved in pathogen-associated molecular pattern identification, among which the Toll-like receptor (TLR) family of at least 10 different members in humans (1, 2) is probably the best known. These receptors are considered sensors for microbial infections or other danger signals and, together with other molecular sensors, serve as a first line of defense, inducing soluble and cellular mediators of innate immunity and initiating key steps of the adaptive immune response (3). Particular clinical interest now revolves around TLR9, which is expressed not only on cells of the immune system but also on endothelial cells, fibroblasts, and epithelial cells (3–6) and which recognizes bacterial and viral DNA with unmethylated CpG motifs. Synthetic oligodeoxynucleotides (ODN) expressing CpG motifs mimic the immunostimulatory activity of bacterial DNA and are commonly used to activate TLR9 for therapeutic applications (3). CpG-ODN has shown antitumor activity in different animal models (7, 8) and in patients with malignant melanoma, renal carcinoma, and recurrent or refractory lymphoma (9–12); however, experimental studies suggest that CpG-ODN may be more useful as a component of multiagent therapy for cancer rather than as a single agent (13). Because chemotherapy is known to be immunosuppressive, it may seem counterintuitive to combine it with TLR9 stimulation. Nevertheless, different chemotherapeutic drugs have been reported to improve the efficacy of CpG-ODN in mouse tumor models (14, 15). In immunocompetent mice, paclitaxel and cyclophosphamide enhanced CpG-ODN effects by depleting regulatory T cells, increasing the immunogenicity of tumor cells, and changing T-cell homeostasis; moreover, the presence of CD8+ T cells was found to be required (16). In immunocompromised athymic mice, the mechanisms...
underlying the improved antitumor effect when CpG-ODN was combined with gemtuzumab (17), gemcitabine (6), or topotecan (18, 19) have to be different from those in immunocompetent mice and remain unclear. It is noteworthy that, although these 3 chemotherapeutic agents differ in mechanisms of action, their cytotoxic activity is generally the consequence of DNA damage. Even ionizing radiotherapy (RT), generally administered locally to the tumor site or draining lymph nodes, kills cancer by damaging DNA, and experiments in murine models suggest that CpG-ODN can enhance the response to RT in both immunogenic (20, 21) and nonimmunogenic tumors (22). For nonimmunogenic tumors, the mechanism remains undefined, as the rationale for even exploring the combination of CpG-ODN with radiation, that is, that dendritic cells acquire antigens released from tumor cells after RT and migrate to regional lymph nodes where they encounter and activate tumor-specific cytotoxic cells, does not apply.

Unlike observations in tumor cells where CpG-ODN seems to increase the activity of RT, studies on the combination of RT and CpG-ODN in mice have shown that TLR9 engagement on CD4+ T lymphocytes reduces apoptosis and enhances their capacity to repair DNA damage induced by γ-radiation (23).

On the basis of these disparate observations, we hypothesized that CpG-ODN modulates genes involved in DNA repair, increasing their expression in TLR9-expressing immune cells but downregulating their expression in tumor cells and thereby increasing sensitivity to DNA-damaging chemotherapeutic agents. To test this hypothesis, we analyzed the effect of CpG-ODN treatment on DNA repair gene expression in immune and tumor cells in vivo and in our previously described model of IGROV-1 ovarian tumor–bearing athymic mice (24) and assessed the antitumor effect of CpG-ODN associated with DNA-damaging chemotherapy in this mouse model.

Materials and Methods

Cell lines

The IGROV-1 tumor (gift from Dr. J. Benard, Institute Gustave Roussy, Villejuif, France) and OVCAR-5 (American Type Culture Collection) were adapted to grow intraperitoneally (i.p.) and maintained by serial intraperitoneal passage in athymic (nude) mice (24). Every 6 months, cells were authenticated by morphologic inspection and by the presence of specific markers with fluorescence-activated cell-sorting analysis. For in vitro experiments, cells were maintained in RPMI-1649 medium supplemented with 10% fetal calf serum (Sigma) and 2 mmol/L glutamine (Cambrex) at 37°C in a 5% CO2 air atmosphere.

In vitro and in vivo experiments for microarray analyses

For microarray experiments, mice were injected i.p. with 2.5 × 106 ascitic cells in 0.2 mL of saline and treated, starting 11 to 12 days later, when mice showed evident and established ascites, with CpG-ODN delivered i.p. at a dose of 20 μg per mouse daily for 3 days. Control mice received saline. Ascites-bearing mice were sacrificed by cervical dislocation 24 hours after the last treatment with saline or CpG-ODN. Tumors adherent to omentum were removed and immediately frozen in liquid nitrogen until RNA or protein extraction.

For microarray experiments, to evaluate a direct action of CpG-ODN on tumor cells, 1 × 106 IGROV-1 cells were seeded in 6-well plates and treated with 10 μmol/L of CpG-ODN in complete culture medium for 24 hours. At the end of treatment, cells were collected and RNA was extracted.

To determine whether local treatment at the tumor site is critical to downregulate DNA repair genes, mice were injected i.p. with IGROV-1 tumor cells as described earlier and treated i.p. or s.c. with CpG-ODN at a dose of 20 μg per mouse daily for 3 days. Twenty-four hours after the last treatment with saline or CpG-ODN, ascites-bearing mice were sacrificed and tumors adherent to the peritoneal wall were removed and immediately frozen in liquid nitrogen until RNA extraction.

Results

DNA repair gene modulation in immune and nonimmune normal cells by CpG-ODN

To evaluate the effect of CpG-ODN on DNA repair genes in immune cells, we conducted a comprehensive in silico expression analysis of genes implicated in DNA repair (GSE11202) in immune spleen cells from mice treated i.p. with CpG-ODN (26). Spleen cells were chosen for this analysis on the basis of previous studies establishing that the spleen accurately reflects the breadth of immunity induced by CpG-ODN in vivo (27–32). Expression levels of mRNA in mouse spleen cells were monitored by microarray at different times after in vivo CpG-ODN treatment. From a list of 209 genes retrieved according to the "DNA repair" term from Gene Ontology (GO:0006281), 189 were present in the GSE11202 and 49 genes were found to be significantly modulated [false discovery rate (FDR) < 0.05] during the course of CpG-ODN treatment, 43 of which were upregulated (Fig. 1). Accordingly, analysis of a published gene expression data set (E-TABM-823) in immune mucosal lung tissue of mice 48 hours after intranasal administration of CpG-ODN, when CpG-ODN-induced recruitment of natural killer and dendritic cell into the bronchoalveolar spaces peaked (33), identified 29 genes in the DNA repair pathway that were significantly modulated by CpG-ODN treatment (FDR < 0.05), 21 of which were upregulated (Supplementary Fig. S1). It is noteworthy that 13 of 29 modulated genes were shared with those modulated in immune spleen cells. Thus, the presence of microbial DNA seems to induce upregulation of genes involved in DNA repair in immune cells.

To test whether CpG-ODN similarly affects nonimmune TLR9-negative normal cells, we conducted expression analysis of DNA repair genes using a whole-mouse genome microarray data set (E-TABM-506), obtained from murine quadriceps muscle injected with CpG-ODN in experiments to evaluate this biodrug as a vaccine adjuvant (34); only 2 genes, NBR and FAM175, for which the specific function of the encoded proteins is unknown, were modulated (FDR < 0.05; data not shown). Thus, CpG-ODN does not induce significant modulation of DNA repair genes in nonimmune normal cells.
The potential effect of CpG-ODN in tumor cells was evaluated on the basis of the expression of the corresponding GO:0006281 mouse genes in a data set obtained from MC38 murine colon carcinoma cells in mice injected intratumorally with CpG-ODN (GSE18203; ref. 35). CpG-ODN treatment was found to modulate DNA repair gene expression in tumors, and 50 genes were modulated at a threshold of $P < 0.05$. However, unlike observations in immune cells, CpG-ODN treatment induced mainly a downregulation of DNA repair genes in tumor cells (40 of 50 modulated genes were downregulated; Fig. 2A and B). These findings were experimentally proofed by microarray analyses of human IGROV-1 ovarian carcinoma xenografts in mice treated daily i.p. with CpG-ODN or saline beginning 3 days after evidence of ascites. Twenty-four hours after the final treatment, tumors adhering to omentum were collected, and RNA extracted from the tumors cells was
analyzed for gene expression profile. Among the 232 genes belonging to GO:0006281 human, 227 genes available in our microarray platform clustered tumors according to saline or CpG-ODN treatment (GSE23441; Fig. 3A), and the pattern of this gene modulation in CpG-ODN–treated mice reflected an increased susceptibility to DNA damage (75 of 114 genes modulated at a threshold of \( P < 0.05 \) were downregulated; Fig. 3B). The power of CpG-ODN in modulating cancer cell DNA repair genes was confirmed by Ingenuity Pathway Analysis (IPA), as IPA of 1,765 up- or downmodulated genes by CpG-ODN compared with controls revealed that the cell death, embryonic development, DNA replication, recombination, and repair pathway were the most influenced (Supplementary Fig. S2). Quantitative real-time PCR (qRT-PCR) of RAD51C, SIRT1, RAD54B, and RAD23B genes validated microarray data (Supplementary Fig. S3).

Results of microarray analyses were also validated by examining the expression of the gene products RAD51 and SIRT1 in tumors from CpG-ODN–treated and control mice. Western blot analysis of tumor cell lysates indicated decreased expression of RAD51 and SIRT1 proteins in treated mice as compared with controls (Fig. 4A and B). Thus, microarray analyses indicate that locally administered TLR9 agonists regulate genes involved in DNA repair in tumor cells in the opposite way from immune cells.

Expression of CpG-ODN–modulated DNA repair genes and survival in ovarian and breast cancer patients treated with DNA-damaging therapy

To evaluate whether CpG-ODN-induced DNA repair gene modulations, observed in IGR0V-1 microarray analysis, were relevant to increase the cell sensitivity to DNA damages, among the genes found differentially modulated between the CpG-ODN–treated and untreated IGR0V-1 tumors, a set of 27 genes with a level of FDR less than 0.01 and a fold difference of more than 1.5 was selected (Supplementary Fig. S4) and the average expression of both CpG-ODN–treated and untreated tumors was calculated for each gene; the resulting expression pattern was used to analyze an ovarian microarray data set containing the gene expression data of ovarian cancer samples obtained at initial cytoreductive surgery from patients who then received platinum-based primary chemotherapy (36). The Pearson correlation coefficient between expression values of the 27 genes and those available in

Figure 3. Microarray analysis of DNA repair pathway genes in IGR0V-1 ovarian tumors from CpG-ODN–treated athymic mice. IGR0V-1–bearing mice with established ascites, that is, increased abdominal volume and body weight, were treated i.p. daily for 3 days with CpG-ODN or saline (control group) and sacrificed 24 hours later. RNA extracted from tumors was analyzed on Illumina human whole-genome bead chips; 227 genes in the DNA repair pathway (GO:0006281 human) were detected in our microarray experiment. A, unsupervised hierarchical clustering of tumors according to expression levels of 227 DNA repair genes. B, heat map of modulated genes. 75 down- and 39 upmodulated (threshold \( P < 0.05 \)) in CpG-ODN–treated mice; (red, upregulated genes; green, downmodulated genes). Each column represents a sample and each row, a gene.
In chemotherapy-treated patients, CpG-like signature was significantly associated with grade \((1 + 2; P = 0.0048)\), whereas no association was found with the other pathologic parameters (estrogen receptor, tumor size, and node status; Supplementary Table S2A). Multivariate analysis of all covariates (i.e., estrogen receptor, tumor size, grade, node status, and CpG-like signature) indicated that CpG-like signature resulted in an independent strong prognostic factor \((HR = 0.3875; 95\% CI, 0.1812–0.8287; P = 0.015)\) of relapse-free survival (Supplementary Table S2B).

Together, these findings indicate that CpG-ODN modulate DNA repair gene expression that are relevant for cell sensitivity to DNA damages.

**Antitumor effect of CpG-ODN and cisplatin in human ovarian tumor xenografts**

To test for a correlation between DNA repair gene down-modulation and sensitivity to DNA-damage-inducing drugs, we evaluated the effect of CpG-ODN treatment on the antitumor activity of cisplatin, a DNA cross-linking agent (38). IGROV-1 ovarian tumor–bearing athymic mice were used in these experiments, as IGROV-1 cells are sensitive to cisplatin (39) and as CpG-ODN in this model has been shown to prolong survival of mice with bulky disease inducing an activation of different effector cells and cytokines of innate immunity at the site of tumor growth (8, 24). Mice were treated i.p. with CpG-ODN, cisplatin, or both 8 days after tumor cell injection, when ascites start to form. Analysis of the effect of the combined treatment revealed a significant \((P < 0.0001)\) increase in life span compared with the use of either reagent alone [treated/control (T/C) values: 200% for cisplatin, 305% for CpG-ODN, and 460% for cisplatin plus CpG-ODN; Fig. 6].

The antitumor efficacy of CpG-ODN in combination with cisplatin was also assayed in mice bearing the human ovarian tumor cells OVCAR-5. As previously observed in IGROV-1 mouse model, mice bearing OVCAR-5 human ovarian cell line and treated with CpG-ODN plus cisplatin survived significantly longer than those treated with CpG-ODN or cisplatin alone (T/C values: 118% for cisplatin, 188% for CpG-ODN, and 267% for cisplatin plus CpG-ODN; Supplementary Fig. S7).

It should be noted that the modulation of DNA repair genes in human ovarian IGROV-1 tumors and the increase in the antitumor effect of cisplatin and CpG-ODN against IGROV-1 human ovarian xenografts in mice were observed in mice injected with a CpG-ODN sequence specific for murine TLR9, making unlikely the possibility that the modulation was related to a direct interaction of CpG-ODN with tumor cells, as different DNA motifs are required for stimulation of mouse and human cells by CpG-ODN (40, 41). Nevertheless, we carried out a microarray analysis on mRNA extracted from IGROV-1 cells stimulated \textit{in vitro} with murine CpG-ODN (GSE23442); none of 16,824 analyzed genes were found to be significantly modulated by CpG-ODN (no genes with FDR < 0.1) compared with IGROV-1 cells cultured in medium alone (data not shown), excluding a direct action of CpG-ODN on tumor cells.

Our evidence of downmodulation in DNA repair genes in tumor cells in the analyses thus far involved the administra-
The injection of CpG-ODN at or near the tumor site is critical for DNA repair gene modulation in tumor cells. Indeed, microarray analyses of mRNA from IGROV-1 tumors adhering to the omentum in mice bearing IGROV-1 ascites and treated subcutaneously daily for 3 days with CpG-ODN revealed no significantly modulated genes (FDR < 0.1) as compared with tumors from saline-treated mice (data not shown); thus, injection of CpG-ODN at the tumor site seems to be critical for DNA repair gene modulation in tumor cells. 

In that case, a relevant role for peritumoral TLR9-expressing cells, such as innate immune cells, and/or endothelial cells, fibroblasts, and epithelial cells, in this modulation is plausible. These cells might induce downregulation of DNA repair genes in tumor cells through a direct cell–cell interaction and/or by secreting soluble factors. 

To evaluate the involvement of soluble molecule(s) in DNA repair gene modulation, cell-free ascitic fluid supernatants, obtained from mice treated i.p. daily for 3 days with CpG-ODN or saline, were added to cultured IGROV-1 cells. Forty-eight hours later, mRNA was extracted and the mRNA levels of *RAD23B*, *RAD51C*, *SIRT1*, and *RAD54B* were determined by qRT-PCR. Incubation with ascitic fluids from CpG-ODN–treated mice induced a downmodulation of *RAD51C* (*P* = 0.0443), *SIRT1* (*P* = 0.0374), and *RAD54B* (*P* = 0.00851) levels, consistent with microarray results, and a trend toward a higher median expression level for *RAD23B* (Supplementary Fig. S8), which was upmodulated in microarray experiments.

Moreover, preincubation of IGROV-1 cells with ascitic fluids from CpG-ODN–treated mice for 48 hours enhanced cisplatin cytotoxicity, as cell viability quantified by MTT assay was 66.0% ± 4.6% (mean ± SEM) for cells preincubated with ascitic fluid from CpG-ODN–treated mice and 90.1% ± 5.8% for cells preincubated with ascitic fluids from saline-treated mice (*P* = 0.0121).

DNA repair gene modulation in tumors by TLR7 agonist imiquimod

Furthermore, to determine whether modulation of DNA repair genes in tumor cells is restricted to CpG-ODN–induced

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**Figure 5.** Association between DNA repair genes modulated by CpG-ODN and survival in patients treated with chemotherapy in an adjuvant setting. Two data sets on whole-genome gene expression profiling of ovarian and breast cancer patients treated with chemotherapy were chosen. A Pearson correlation coefficient was calculated to assess the correlation between each tumor and the CpG-ODN signature. The correlation was determined on the basis of expression values of the 27 genes found modulated, comparing the CpG-ODN treatment versus the control condition, and the corresponding genes available in each data set. Tumors were split into 2 groups according to the difference between the correlation obtained for the CpG-ODN treatment and the control condition, with the average used as threshold. Kaplan–Meier curves indicate the survival probability for patients with ovarian cancer (A), patients with breast cancer who received adjuvant systemic chemotherapy (B), and patients with breast cancer who did not receive any systemic treatment (C). Patients with a greater correlation to the CpG-ODN than with the untreated condition showed better outcome. Black curve, CpG-like patients; gray curve, CpG-untreated-like patients.
TLR9 activation or occurs after activation with the only other TLR agonist currently used in oncologic therapy, imiquimod—an immunomodulator that functions as agonist of TLR7 and/or TLR8 (42)—a comprehensive in silico expression analysis of genes implicated in DNA repair (GO:0006281 mouse) on mRNA extracted from spontaneous s.c. tumors from mice treated topically with imiquimod (GSE20032; ref. 43) was conducted. Imiquimod treatment was found to modulate DNA repair gene expression in tumors, which clustered according to treatment (treated vs. control; Supplementary Fig. S9A), and the pattern of gene modulation in imiquimod-treated mice reflected an increased susceptibility to DNA damage (29 of 32 genes modulated at a threshold of $P < 0.05$ were downregulated; Supplementary Fig. S9B).

**Discussion**

In the present study, we show by microarray analyses that TLR9 ligand CpG-ODN treatment induces downmodulation of DNA repair genes in tumor cells of both murine and human origins. Expression-level analysis of proteins, RAD51, a key protein in the homologous recombination DNA repair pathway (44), and SIRT1, whose activity promotes homologous recombination (45), in human tumor cells confirmed microarray results. These proteins are involved in homologous recombination and, consequently, are relevant for the repair of interstrand cross-links, which are the most cytotoxic lesions induced by cisplatin. Accordingly, the combination of cisplatin and CpG-ODN against IGROV-1 human ovarian tumor xenografts in athymic mice was found to induce a remarkable increase in life span compared with that using either reagent alone ($P < 0.0001$).

The combined data from our analysis and in silico analyses revealed modulation of DNA repair genes in both murine colon carcinoma MC38 cells and human ovarian carcinoma IGROV-1 cells, suggesting that this effect is not restricted to a specific cancer cell histotype. The observation that this modulation also occurs after treatment with an agonist of TLR7 and/or TLR8 might suggest that this event can be shared by the functional subgroup of TLRs, consisting of TLR7, TLR8, and TLR9, which reside intracellularly and recognize nucleic acids derived from the genome of viruses and bacteria (42).

The findings that intratumoral delivery of CpG-ODN was critical in inducing DNA repair protein downmodulation in tumors and that CpG-ODN did not interact directly with the tumor cells to induce this downmodulation in IGROV-1 ovarian cancer cells point to the importance of the activation of the TLR9-positive cells (innate immune cells, endothelial cells, fibroblasts, and epithelial cells) present in the tumor microenvironment, in modulating DNA repair gene expression. Moreover, our data suggest that soluble molecules in ascitic fluid, presumably released by the above-mentioned TLR9-positive cells, are involved in the modulation of DNA repair genes. The lower modulation of DNA repair genes observed in ex vivo experiments compared with that in in vivo might depend on the loss of activity of some factors or on the need for both soluble factors and contact between CpG-ODN-activated cells and tumor cells.

Clinical results from association of CpG-ODN with DNA-damaging drugs in lung tumor patients have so far been disappointing in improving clinical outcome (46).

On the basis of our findings, this absence of CpG-induced enhanced chemosensitivity of tumor cells to DNA-damage-inducing agents may rest in the s.c. administration of CpG-ODN, distant from the tumor cells.

On the contrary, we observed by in silico analyses that TLR9 ligand CpG-ODN treatment induces essentially an upmodulation of DNA repair genes in immune cells. Moreover, it is noteworthy that 19 of 49 genes modulated in spleen cells after CpG-ODN treatment and even did so in IGROV-1 tumor cells, but for most of them the modulation was in an opposite way (14 genes upregulated in spleen were downregulated in tumor, and 3 genes downregulated in spleen were upregulated in tumor).

Despite numerous previous microarray analyses of immune cells after in vitro or in vivo stimulation with CpG-ODN (26, 33, 35, 43, 47), to our knowledge, an upregulation of DNA repair genes after CpG-ODN treatment has not been reported. This might reflect a primary focus in previous studies on signaling pathways that induce expression of immune and proinflammatory genes (48), in light of the function of TLRs as innate immunity sensors of microbial products.

CpG-ODN–induced downmodulation of DNA repair genes in tumor cells and upmodulation in immune cells might represent a physiologic phenomenon that occurs locally in the presence of an infectious event. Thus, upon detecting the presence of an infectious agent via endosomal TLRs, immune cells might regulate DNA repair genes to decrease their susceptibility to possible proapoptotic signals during infections and, at the same time, directly and/or indirectly induce...
modulation of DNA repair genes in infected (or transformed) cells to facilitate their death. Insignificant modulations of these genes observed in normal muscle cells suggest that CpG-ODN–activated immune cells induce downmodulation of DNA repair genes only in “altered” cells expressing aposite receptors. Moreover, better outcome of cisplatin-treated ovarian carcinoma patients, as well as of anthracycline-treated breast cancer patients, classified as CpG-like, compared with patients classified as CpG-ununtreated-like, indicates the relevance of these genes in the tumor cell response to DNA-damaging drugs. In subjects who did not receive adjuvant chemotherapy, expression of CpG-ODN–modulated DNA repair genes was not associated with significantly better outcome, indicating that these genes are not prognostic of survival.

Together, our present data provide the first evidence that TLR9-expressing cells present in the tumor microenvironment can sensitize cancer cells to DNA-damaging chemotherapy, underscoring the need for further investigation of the synergistic effect of CpG-ODN in combination with DNA-damaging drugs in cancer treatment.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest.

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