CpG Immunomer DNA Enhances Antisense Protein Kinase A Rlα Inhibition of Multidrug-Resistant Colon Carcinoma Growth in Nude Mice: Molecular Basis for Combinatorial Therapy

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Abstract

**Purpose:** CpG DNAs induce cytokines, activate natural killer cells, and elicit vigorous T-cell response leading to antitumor effects. Antisense oligodeoxynucleotides targeted against the Rlx subunit of protein kinase A (antisense PKA Rlx) induce growth arrest, apoptosis, and differentiation in a variety of cancer cell lines in vitro and in vivo. This study investigated the use of a combinatorial therapy consisting of the RNA-DNA second-generation antisense PKA Rlx and the CpG immunomer (CpG DNA linked through 3'-3' linkage containing two accessible 5' ends).

**Experimental Design:** HCT-15 multidrug-resistant colon carcinoma growth in nude mice was used as an experimental model. The inhibitory effect on tumor growth and apoptotic activity of antisense Rlx and CpG immunomer, singly and in combination, were measured by tumor growth, levels of Rlx subunit, and antiapoptotic and proapoptotic proteins. Effect on host-immune system was measured by mouse spleen size, interleukin-6 (IL-6) levels in mouse blood, and nuclear factor-κB (NF-κB) transcription activity in mouse spleen cells.

**Results:** In combination, CpG immunomer and antisense PKA Rlx induced additive/supra-additive effect on the inhibition of tumor growth. Antisense Rlx but not CpG immunomer increased Bax and Bak proapoptotic protein levels and decreased Bcl-2 and Rlx protein levels in tumor cells. CpG immunomer but not antisense Rlx induced an enlargement of mouse spleen, increased IL-6 levels in mouse blood, and increased NF-κB transcription activity in mouse spleen cells.

**Conclusions:** These results show that type 1 PKA down-regulation and induction of apoptosis in tumor cells by antisense PKA Rlx, and host-immune stimulation by CpG immunomer are responsible at the molecular level for the supra-additive effects of tumor growth inhibition. Thus, antisense PKA Rlx and CpG immunomer in combination work cooperatively and as tumor-targeted therapeutics to treat human cancer.

Malignant tumor growth and response of tumors to various therapeutic modalities are thought to be affected by the host-immune system. Tumor development and growth may be facilitated when the host-immune system is deficient, whereas they may be restricted when the immune system is fully stimulated (1). If so, conventional cancer therapies may be more effective under the conditions where the host-immune system is stimulated (1).

Almost two decades ago, it was shown that bacteria, especially *Mycobacterium bacillus* Calmette Guerin could cause regression of tumor growth (2, 3). One of the active components of *Mycobacterium bacillus* Calmette Guerin responsible for natural killer cell activation and tumor regression is its genetic material DNA (4, 5). Later, bacterial DNA was also shown to induce B-cell proliferation and immunoglobulin production, whereas mammalian DNA does not (6, 7). The explanation of this phenomenon was found in the observation that mammalian and bacterial DNA reveals different structure. CpG dinucleotides are generally present at the expected frequency of 1 per 16 dinucleotides in microbial DNA, but they are only about one quarter as prevalent in vertebrates (7, 8). Moreover, the cytosines in CpG dinucleotides are highly methylated in vertebrates but not in microorganisms (8).

Later, it was shown that CpG DNAs containing different sequences and structures could stimulate specific subsets of immune cells that produce different types of immune responses and cytokine activation profiles (9, 10). The unique properties of CpG DNAs opened the possibility of using them for modulating certain “desired” immune responses to treat various diseases including cancer. Recently, it was shown that CpG DNAs linked through 3'-3' linkage containing two accessible 5' ends, referred to as immunomers, have enhanced activity (11, 12), and synthetic CpR (R = 2'-deoxy-7-deazaguanosine) dinucleotide shows potent immune stimulatory activity with distinct cytokine secretion patterns (11–13). Immunopharmacologic and antitumor properties of these second-generation immunomodulatory oligonucleotides (CpG immunomer) were evaluated in vivo either alone or in combination with chemotherapeutic agents (14). Repeated administration of
CpG immunomers to mice bearing established CT26 colon tumor and B16.F0 melanoma resulted in strong inhibition of tumor growth. Additionally, the coadministration of CpG immunomers with chemotherapeutic agents, docetaxel and doxorubicin, resulted in synergistic anti-tumor effects.

However, to our knowledge, the combined effect of CpG DNAs with antisense oligonucleotides has not been explored. In principle, an antisense oligodeoxynucleotide targeted against a gene involved in the neoplastic cell growth should interfere with that gene expression, resulting in arrest of cancer cell growth (15). Protein kinase A type I (PKA-I) plays a key role in neoplastic transformation (16). It conveys mitogenic signals from different sources (17, 18) and is overexpressed in the majority of clinical tumors examined (19), and down-regulation of PKA-I results in tumor cell growth inhibition (20). Our laboratory has been engaged with the antisense studies targeted against the Rαs regulatory subunit of PKA-I (antisense Rαs) and has shown the down-regulation of Rαs and inhibition of tumor cell growth in vitro and in vivo (21–24).

We and others, using the second-generation antisense Rαs of RNA-DNA mixed backbone oligodeoxynucleotide, have shown further that the second-generation antisense Rαs compared with the first-generation antisense oligodeoxynucleotide (i.e., PS-ODN) exhibits improved pharmacokinetic properties and antitumor activity accompanied by an increase in apoptosis in human cancer cell lines (24–27).

Cytotoxic drugs and radiotherapy usually damage normal cells at the conditions where there is no complete killing of cancer cells. In this regard, targeted drugs, such as antisense oligodeoxynucleotides that can selectively block cancer-causing genes and preferentially kill cancer cells, would be desirable to treat cancer. Thus, antisense oligodeoxynucleotides that selectively kill cancer cells, in combination with CpG DNAs that stimulate the host-immune system would enhance antitumor effect but with minimum adverse effects on normal cells. In the present study, we therefore investigated whether antisense Rαs and immunomodulating CpG DNA can produce cooperative effect on tumor growth inhibition. We used the second-generation mixed backbone antisense PKA Rαs and CpG immunomer in the present study.

Materials and Methods

Chemicals. Monoclonal antibody for PKA Rαs subunit was purchased from BD Biosciences (San Diego, CA). Polyclonal antibodies for Bcl-2, Bak, and Bax were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Immunoassay kit for mouse interleukin-6 (IL-6) was purchased from BioSource International, Inc. (Camarillo, CA).

Oligonucleotides. The second-generation RNA-DNA mixed backbone antisense Rαs oligodeoxynucleotide used in the study is 5’-[GGCU][GCCCTCTTCAG][UGGC]-3’, targeted against the NH2-terminal 8 to 13 codons of the human Rαs regulatory subunit of PKA (24). This oligodeoxynucleotide is a PS-DNA antisense containing segments of 8 to 13 codons of the human RI regulatory subunit was 5’-[NNNN][NNNNNNNNNN][NNNN]-3’, a random sequence oligonucleotide, with the bracketed segments representing 2’-O-methyl-RNA and N representing A, T, C, or G.

The CpG immunomer is 5’-[TCTGACGTTCT-X-TCTFGCACTG]-3’ where X is a glycerol linker, and the non-CpG control DNA, 5’-CATATCACCCTTCTCGT-3’ were synthesized, purified, and analyzed as previously described (11). These antisense and CpG oligonucleotides were kindly provided by Dr. S. Agrawal (Hybridon, Inc., Cambridge, MA).

Cell culture. HCT-15 colon carcinoma cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotic-antimycotic in a humidified atmosphere of 95% air and CO2 at 37°C.

Tumor growth and oligonucleotide treatment. HCT-15 human colon carcinoma cells (2 × 10⁶ cells) were inoculated s.c. into the left flank of athymic mice. Rαs antisense and control oligodeoxynucleotides were injected i.p. daily at the concentration of 0.1 mg in 0.1 mL saline per mouse. CpG immunomer and control CpG DNA were injected s.c. in the right flank of mice daily at the concentration of 0.01 mg in 0.1 mL saline per mouse. Oligodeoxynucleotides were injected when tumor size reached 30 to 40 mg. 1 week after cell inoculation. Tumor volumes were obtained from daily measurement of the longest and the shortest diameters and calculation by the formula 4/3 × πr² where r = (length + width) / 4 (23). At the end of the experiment, animals were sacrificed.

Western blot analysis. Proteins were separated on 12% SDS-polyacrylamide gel, and separated proteins were transferred onto nitrocellulose membrane using semidybrid blotting system. Anti-Rαs, Bcl-2, Bak, and Bax antibodies for Bcl-2, Bak, and Bax were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The homogenate were centrifuged at the maximum speed of Eppendorf Centrifuge for 10 minutes, and the resulting supernatants used as cytosol. Protein concentration was determined by the method of Lowry et al. (28) using bovine serum albumin as standard.

Electrophoretic mobility shift assay. Nuclear extracts were prepared by the method of Dignam et al. (29). Electrophoretic mobility shift assay was done by the method of Fried and Crothers (30). Briefly, nuclear extracts (5 μg of protein) were combined with poly(deoxyninosinic-deoxy cytidylic acid) (2 μg), DT (0.3 mmol/L), reaction buffer [12 mmol/L Tris (pH 7.9), 2 mmol/L MgCl₂, 60 mmol/L KCl, 0.12 mmol/L EDTA, and 12% glycerol], and 32P-labeled oligonucleotides (double-stranded oligonucleotides with one copy of nuclear factor-κB (NF-κB), 5’-AGTTGAGGGCACTTCCCCAGG-3’ (Promega, Madison, WI) and the reaction mixtures were incubated for 30 minutes at room temperature. The reaction mixtures were separated on a 4% nondenaturing polyacrylamide gel at 200 V for 2 hours. The gel was dried and autoradiographed.

Results

Combination treatment of antisense Rαs oligodeoxynucleotide and CpG immunomer to inhibit tumor growth. In our previous studies, we have shown that PKA Rαs antisense produced growth inhibition of tumors in vivo and cancer cells in culture (23, 24). Among these cell lines were HCT-15 colon carcinoma cells, well known for high level of multidrug resistance. In the present study, we used this cell line to produce tumors in nude mice. HCT-15 human colon carcinoma cells (2 × 10⁶ cells) were
inoculated s.c. into the left flank of athymic mice. RIA antisense oligodeoxynucleotide and control oligodeoxynucleotide were injected i.p daily 0.1 mg/mouse, and CpG immunomer and control CpG were injected s.c. daily, 0.01 mg/mouse starting when tumors were just palpable. Both antisense RIA and CpG immunomer exhibited growth inhibitory effect within 1 week posttreatment, and by 3 weeks, each oligodeoxynucleotide produced 50% to 60% growth inhibition (Fig. 1). By contrast, control antisense oligodeoxynucleotide and control CpG exhibited no growth inhibitory effect (Fig. 1). Strikingly, when antisense RIA and CpG immunomer were given together, there occurred over 90% of growth inhibition by 3 weeks of treatment (Fig. 1). Thus, antisense RIA and CpG immunomer in combination acted cooperatively, producing additive or supra-additive inhibitory effects on tumor growth in vivo.

Splenomegaly in vivo. In general, the increase in spleen weight (splenomegaly) of mice injected with CpG DNAs is considered as a result of the immunostimulatory activity of CpG DNA (31, 32). To examine that CpG immunomer acted as an immunostimulatory agent in vivo, we examined at the end of experiment the weight of mouse spleen (Fig. 2). Our results show that tumor itself can cause a small increase in spleen size (Fig. 2). Administration of saline, antisense RIA, or control oligodeoxynucleotides did not further affect the size of mouse spleen. The injections of CpG immunomer alone or in combination with other oligodeoxynucleotides under study stimulated spleen enlargement about 2-fold (Fig. 2).

Interleukin-6 secretion in vivo. We next measured the cytokine IL-6 levels in mouse serum, as the induction of IL-6 has been used as a hallmark for CpG immune stimulation. CpG immunomer showed the marked increase of IL-6 level in mouse blood (Fig. 3). RIA and control oligodeoxynucleotides did not affect IL-6 levels in the serum of tumor-bearing mice (Fig. 3).

Activation of nuclear factor-κB transcription in spleen in vivo. CpG DNA recognition by Toll-like receptor-9 (TLR-9) has been shown to activate a number of signaling pathways, including the NF-κB pathway, which plays a critical role in the up-regulation of cytokine gene expression (33). To study the effect
of antisense oligodeoxynucleotide and CpG immunomer on activation of NF-κB in vivo, we prepared nuclear extract from the spleen of animals under experiment and examined it by electrophoretic shift mobility assay for NF-κB activation. The results are presented in Fig. 4. The administration of CpG immunomer led to a marked increase of NF-κB activation. The antisense RIα and control oligodeoxynucleotides failed to increase NF-κB activation in mouse spleen.

**Effect of antisense RIα and CpG immunomer on RIα and apoptotic protein expression.** We measured the effect of antisense RIα oligodeoxynucleotide on the expression of the targeted gene product RIα by Western blotting analysis (Fig. 5). The level of RIα protein was markedly down-regulated in tumor extracts prepared from the antisense RIα-treated animals. Control oligodeoxynucleotides and CpG immunomer treatment did not affect the level of RIα in tumor cells.

Several recent studies have linked PKA to the apoptotic machinery (25, 26). In the present study, we observed the decrease in antiapoptotic Bcl-2 protein and increase in Bak and Bax proapoptotic proteins in tumor extract from animals injected with antisense RIα alone and in combination with CpG immunomer (Fig. 5). Control oligodeoxynucleotides and CpG immunomer alone did not affect the levels of these proteins in HCT-15 tumors (Fig. 5).

**Discussion**

Nucleic acid therapeutics represent a direct genetic approach to cancer treatment. Such methodology takes advantage of genes known to confer a growth of neoplastic cells and mechanisms that activate these genes (15). Antisense technology is historically the first nucleic acid-based approach for cancer therapy. Use of this method with correct choice of target would allow achieving better success in the eradication of cancer.

Cyclic AMP-dependent protein kinase type I (PKA-I) mediates mitogenic signals from different growth factors, plays a role in the G1-S transition in the cell cycle, and functions in cell proliferation and neoplastic transformation (16–18, 34). An antisense strategy has been used to explore a potential role for the RI subunit of PKA-I as a positive regulator that is essential for cancer cell growth. Antisense oligodeoxynucleotides targeted to the NH2 terminus of the RIα subunit not only

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**Fig. 3.** *In vivo* IL-6 induction by CpG-IMO. Sera were collected at the end of experiment and IL-6 levels determined by ELISA following manufacturer’s protocol (BioSource International). See Fig. 2 legend for the treatment of animals with oligonucleotides. Representative of one of two separate experiments that gave similar results.

**Fig. 4.** Effect of CpG-IMO on NF-κB activation in spleen in vivo. At the end of the experiment, spleen was removed and nuclear extract prepared and analyzed on native polyacrylamide gel as described in Materials and Methods. Nuclear extracts were prepared from spleen of animals treated with saline (lane 2); CpG immunomer, CpG (lane 3); CpG, competitor added at EMSA (lane 4); antisense RIα, ASR Iα (lane 5); AS RIα + CpG (lane 6); control oligodeoxynucleotide, CODN (lane 7); CODN + CpG (lane 8); and control CpG, CCpG (lane 9). Representative of one of three separate experiments that gave similar results.
inhibited PKA-I expression but also exhibited antitumor activity in nude mice (23, 24). The Rlx antisense selectively inhibited Rlx expression, leading to up-regulation of the RIIβ subunit (22–25, 35, 36). Such up-regulation was attributable to an increase in the stability of the RIIβ protein due to its holoenzyme formation in PKA-II, that ensured the depletion of PKA-I and the sustained inhibition of tumor growth (23, 36). The Rlx antisense oligodeoxynucleotide seemed to restrain tumor cell growth by inhibiting tyrosine kinase signaling, cell cycle deregulation, and induction of apoptosis and differentiation (25, 36–40). Treatment with Rlx antisense of different types of cancer cell lines (MCF-7 hormone dependent, MDA-MB-231 hormone-independent breast cancer, PC3M hormone-insensitive prostate cancer) induces growth inhibition through apoptotic pathway (25, 38, 40). Along with these data, our present study shows that antisense Rlx inhibits HCT-15 multidrug resistant tumor growth in nude mice involving modulation of apoptotic proteins, such as Bcl-2, Bax, and Bak.

Another type of therapeutic oligonucleotides used in this study was a synthetic CpG-containing DNA CpG immunomunoster. Bacterial and synthetic CpG DNA have a mitogenic effect on B cells, activate macrophages, dendritic cells, monocytes to produce cytokines, and stimulate natural killer cell lytic activity (7, 41–43). In vivo, these motifs induce splenomegaly accompanied by proliferation of splenic B cells, increased expression of MHC class II on B cells, increased synthesis of RNA and DNA, and increased number of immunoglobulin-producing cells (31, 32).

Immunomodulatory CpG-containing oligonucleotides can produce antitumor effects (14). In the present study, we showed that the CpG immunomunoster produced antitumor effects on the multidrug resistant HCT-15 tumor growth in nude mice and induced splenomegaly, increased IL-6 production, and increased NFκB transcription activity in mouse spleen cells. The mechanism of antitumor action of CpG-DNAs may be entirely different from that of antisense oligodeoxynucleotides. The antitumor action of antisense Rlx involves induction of apoptosis, which is through the caspase-dependent pathway, whereas the recognition of CpG DNA by immune cells occurs through a transmembrane protein that belongs to the TLR family (44). The TLR family proteins recognize conserved pathogen-associated molecular patterns that are not present in the host, which results in the activation of antipathogenic responses. A recently discovered TLR family protein, TLR9, recognizes unmethylated CpG dinucleotides in specific sequence contexts present in bacterial and synthetic DNA (44). This signaling event leads to the activation of NFκB system and up-regulation of various cytokines which agree with the results obtained in this study.

Thus, antisense Rlx oligodeoxynucleotide that induces cell apoptosis and differentiation and CpG immunomunoster that stimulates host-immune system can produce a supra-additive antitumor effect through activation of different cell signaling pathways. Importantly, a recent report suggested the possible crosstalk between these two types of nucleic acid medicines (45). They investigated the cytokine-induced cell death, which is a caspase-independent process and is characterized by increased production of reactive oxygen species in the mitochondria. They showed that the tumor necrosis factor–induced an increased phosphorylation of glyoxalase I that is mediated by protein kinase A is critically required for the cell death. Thus, antisense Rlx can play a critical role in the antitumor action of immunostimulatory CpG DNAs. Our results suggest that combined treatment of tumors with antisense Rlx and a CpG DNA can be promising to achieve the tumor-targeted therapeutic approach for treatment of human cancer.

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References

9. Verthelyi D, Ishii KJ, Gursel M, Takeshita F, Klinman...


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