Antisense Bcl-2 Oligodeoxynucleotides Inhibit Progression to Androgen-Independence after Castration in the Shionogi Tumor Model

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ABSTRACT

Progression to androgen-independence remains the main obstacle to improving survival for patients with advanced prostate cancer. Although Bcl-2 expression in normal prostate epithelial cells is low or absent, Bcl-2 is highly up-regulated in prostate cancer cells after androgen withdrawal and during progression to androgen-independence. Here, we test the efficacy of antisense Bcl-2 oligodeoxynucleotide (ODN) therapy administered adjutively after castration to delay time to androgen-independent recurrence in the androgen-dependent mouse Shionogi tumor model. Treatment of Shionogi tumor cells in vitro with antisense Bcl-2 ODN inhibited Bcl-2 expression in a dose-dependent and sequence-specific manner. Systemic administration of antisense Bcl-2 ODN in mice bearing Shionogi tumors beginning 1 day postcastration resulted in a more rapid regression of tumors and a significant delay of emergence of androgen-independent recurrent tumors. Furthermore, despite significant reduction of Bcl-2 expression in tumor tissues, antisense Bcl-2 ODN had no effect on Bcl-2 expression in normal mouse organs. These findings illustrate the potential utility of antisense Bcl-2 therapy for prostate cancer in an adjutant setting with androgen ablation.

INTRODUCTION

No therapy exists that is superior to androgen ablation in patients with advanced prostate cancer. Approximately 80% of patients achieve symptomatic and/or objective response after androgen ablation; however, progression to androgen-independence ultimately occurs and remains the main obstacle to improving the survival and quality of life in this disease (1). To date, new nonhormonal therapies have been evaluated in patients with hormone refractory disease. When used in this end-stage setting, no nonhormonal agents have improved survival (2). A more rational strategy, therefore, would involve earlier application of novel agents to delay the emergence of the AI phenotype.

bcl-2, first recognized as the proto-oncogene translocated to the immunoglobulin heavy-chain locus in human B-cell lymphoma cells, is the prototype of a novel class of oncoproteins that contributes to neoplastic progression, not by accelerating cell proliferation but rather by enhancing tumor cell survival through the inhibition of apoptosis (3). In prostate cancer, experimental and clinical observations strongly suggest that Bcl-2 plays a critical role in the progression to androgen-independence through the inhibition of apoptotic cell death precipitated by androgen ablation (4–9). Bcl-2 overexpression is also associated with resistance to several cytotoxic chemotherapies and radiotherapy (6, 7, 9).

The controlled study of the complex molecular processes associated with AI progression has been difficult because there is no ideal animal model that mimics the clinical course of the disease in men. The AD Shionogi mouse mammary carcinoma model is particularly useful to assess the influence of androgen ablation on several molecular events during AI progression, including gene expression associated with apoptotic cell death. In this model, AD tumors in intact mice undergo complete regression after androgen withdrawal, but rapidly growing AI tumors recur after 1 month in a highly reproducible manner (10).

Antisense ODNs are chemically modified stretches of single-stranded DNA that are complementary to mRNA regions of a target gene and effectively inhibit gene expression by forming RNA/DNA duplexes (11). Phosphorothioate ODNs are stabilized to resist nuclease digestion by substituting one of the nonbridging phosphor oxygens of DNA with a sulfur. Recently, several antisense ODNs specifically targeted against genes involved in neoplastic progression have been evaluated both in vitro and in vivo as potential therapeutic agents (12–15). In the present study, based on the accumulating evidence implicating Bcl-2 in AI progression, we tested whether the adjvant use of antisense Bcl-2 ODN with androgen ablation enhances castration-induced apoptosis and delays progression to AI in the Shionogi tumor model.

MATERIALS AND METHODS

Antisense Bcl-2 ODN. Phosphorothioate ODNs used in this study were generously supplied by Dr. Brett P. Monia at ISIS Pharmaceuticals (Carlsbad, CA). The sequences of antisense Bcl-2 ODN corresponding to the mouse bcl-2 translation initiation site were 5'-TCTCCCGGTTGGCGCCAT-3'. Two base Bcl-2 mismatch ODNs (5'-TCTCCGGCATGTGCGCCAT-3') were used as control.

Shionogi Tumor Growth. The Toronto subtype of the transplantable SC-115 AD mouse mammary carcinoma (16) was used in all of the experiments. Shionogi tumor cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% heat-inactivated FCS. For in vivo study, approximately 5 x 10^6 cells of the Shionogi carcinoma were injected s.c. into adult male DSS strain mice. When Shionogi tumors became 1 to 2 cm in diameter, usually 2–3 weeks after injection, castration was performed through an abdominal incision under methoxyflurane anesthesia. Details of the maintenance of mice, tumor stock, and operative procedures were described previously (17).

Treatment of Cells with ODN. Lipofectin, a cationic lipid (Life Technologies, Inc., Gaithersburg, MD) was used to increase the ODN uptake of cells. Shionogi cells were treated with various concentrations of ODN after a preincubation for 20 min with 4 µg/ml lipofectin in serum-free OPTI-MEM (Life Technologies, Inc.). Four h after the beginning of the incubation, the medium containing ODNs and lipofectin was replaced with the standard medium described above.

Northern Blot Analysis. Total RNA was isolated from cultured Shionogi tumor cells and Shionogi tumor tissues by the acid-guanidium thiocyanate-phenol-chloroform method. Poly(A)^+ mRNA was then purified from total RNA using oligo(dexoxythymidylate) cellulose (Pharmacia Biotech Inc., Uppsala, Sweden). Five µg of poly(A)^+ mRNA from each sample was subjected to electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nylon membranes overnight according to standard procedure (18). The RNA blots were hybridized with a mouse Bcl-2 cDNA probe labeled with [32P]jdCTP by random primer labeling. After stripping, the membranes were rehybridized with the probe described above.

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3 The abbreviations used are: ODN, oligodeoxynucleotide; AD, androgen-dependent; AI, androgen-independent; PARP, poly(ADP-ribose) polymerase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.
with a mouse G3PDH cDNA probe. The density of bands for Bcl-2 was normalized against that of G3PDH by densitometric analysis.

**Western Blot Analysis.** Samples containing equal amounts of protein (15 μg) from lysates of the cultured Shionogi cells and Shionogi tumors were electrophoresed on a SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The filters were blocked in PBS containing 5% nonfat milk powder at 4°C overnight and then incubated for 1 h with a 1:200-diluted C-2, an antihuman Bcl-2 monoclonal antibody that reacts with mouse Bcl-2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), 1:10,000-diluted MAB065, an antirat β-tubulin mouse monoclonal antibody (Chemicon International Inc., Tumecula, CA) that reacts with mouse β-tubulin, or 1: 600-diluted C2–10, an antihuman PARP mouse monoclonal antibody that reacts with mouse PARP (Pharmingen, Mississauga, Ontario, Canada). The filters were then incubated for 30 min with horseradish peroxidase-conjugated antismouse IgG antibody (Amersham Life Science, Arlington Heights, IL), and specific proteins were detected using an enhanced chemiluminescence Western blotting analysis system (Amersham Life Science).

**Assessment of in Vivo Tumor Growth.** Male C3H/He mice bearing Shionogi tumor were castrated and randomly selected for treatment with antisense Bcl-2 versus mismatch control ODN. Each experimental group consisted of seven mice. One day after castration, 12.5 mg/kg antisense Bcl-2 or mismatch control ODNs, diluted with PBS, were administered by i.p. injection into each mouse once daily for 40 days. Tumor volume was measured twice weekly and calculated by the formula, length × width × depth × 0.5236 (19). Data points were reported as average tumor volumes ± SD.

**RESULTS**

**Changes of Bcl-2 mRNA Expression in the Shionogi Tumor Model.** Northern blot analyses were used to characterize changes in Bcl-2 mRNA expression in AD intact tumors before castration, in regressing tumors 4 and 7 days after castration, and in AI recurrent tumors 28 days after castration. As shown in Fig. 1. A and B, Bcl-2 mRNA expression was up-regulated 5-fold and 3-fold 4 and 7 days after castration, respectively, and was maintained at 2-fold higher levels in AI tumors than in AD intact tumors before castration. The pattern of Bcl-2 up-regulation in the Shionogi tumor model during AI progression is similar to that in the clinical disease (8, 9) and, therefore, supports the use of this model to evaluate the effect of adjuvant antisense Bcl-2 therapy on progression to AI.

**Antisense ODN-mediated Inhibition of Bcl-2 Expression in Shionogi Tumor Cells.** The effect of treatment with antisense Bcl-2 ODN on Bcl-2 mRNA expression in Shionogi tumor cells was initially evaluated by Northern blot analysis. As shown in Fig. 2, A and B, daily treatment of Shionogi tumor cells with antisense Bcl-2 ODNs (50, 100, 500, or 1000 nm) for 2 days reduced Bcl-2 mRNA levels by 5, 25, 77, or 94%, respectively. In contrast, Bcl-2 mRNA expression was not affected by the two-base mismatch control ODNs at any of the used concentrations.

To further analyze the specificity of antisense Bcl-2 ODNs, Northern blotting was performed on other apoptosis-associated genes, bax and bcl-xL, both of which share significant sequence homology with bcl-2. Antisense Bcl-2 ODNs markedly reduced Bcl-2 mRNA expression, but no effects were observed on Bax and Bcl-xL expression levels (Fig. 2C). These findings demonstrate that antisense Bcl-2 ODNs used in these studies do not effect expression of related isotypes.

To determine whether the decrease in Bcl-2 mRNA levels induced by antisense ODN is accompanied by a corresponding decrease in protein levels, Western blot analysis was used to measure changes in Bcl-2 protein levels in Shionogi tumors cells after daily treatment with antisense Bcl-2 ODNs for 4 consecutive days. Dose-dependent inhibition of Bcl-2 protein levels was observed with antisense Bcl-2 but not with mismatch control ODN treatment (Fig. 3).

**Delayed AI Progression of Shionogi Tumors by Antisense Bcl-2 ODN Treatment.** Male mice bearing Shionogi tumors were castrated 2 to 3 weeks after tumor implantation, at which time tumors were 1 to 2 cm in diameter, and randomly selected for treatment with antisense Bcl-2 versus mismatch control ODN. Mean tumor volume was similar in both groups at the beginning of ODN treatment. Beginning 1 day after castration, 12.5 mg/kg ODN was administered once daily by i.p. injection for 40 days. As shown in Fig. 4, Shionogi tumors regressed faster and complete regression occurred earlier in mice treated with antisense Bcl-2 ODN compared to those treated with mismatch control ODN. Furthermore, antisense Bcl-2 ODN treatment significantly delayed recurrence of AI tumors compared to mismatch control ODN treatment. During an observation period of 50 days postcastration, AI tumors recurred in 5 of 7 mice after a median of 44 days in antisense Bcl-2 ODN treatment group, while AI tumors recurred in all of the mice after a median of 29 days in mismatch control ODN treatment group. Under the experimental conditions used in the above in vivo experiment, no side effects associated with antisense Bcl-2 or mismatch control ODN treatment were observed.

We then examined the effects of in vivo ODN treatment on Bcl-2 mRNA expression in Shionogi tumors by Northern blotting. In this experiment, beginning 1 day postcastration, each of three tumor-bearing mice were given 12.5 mg/kg antisense Bcl-2 or mismatch control ODN i.p. once daily, and tumor tissues were harvested for RNA extraction 4 days after castration. Antisense Bcl-2 ODN resulted in a 72% reduction in Bcl-2 mRNA levels in Shionogi tumors compared with mismatch control ODN-treated tumors (Fig. 5. A and B).

To determine whether more rapid regression of antisense ODN-treated tumors resulted from an earlier onset of castration-induced apoptosis, Western blotting of tumor tissues was used to measure the cleavage of PARP protein, a substrate of the caspase activated during the final process of apoptotic execution (20). Proteins were extracted 4 days postcastration from each of three Shionogi tumors in mice given antisense Bcl-2 or mismatch control ODNs under the same
treatment schedule described above. The Mr 116,000 intact form of PARP was observed in both antisense Bcl-2 ODN-treated and mismatch control ODN-treated Shionogi tumors, whereas the Mr 85,000 PARP cleavage fragment was clearly detectable only in antisense Bcl-2 ODN-treated Shionogi tumors (Fig. 6).

We also evaluated changes in Bcl-2 mRNA levels in various normal mouse organs reported to express detectable levels of Bcl-2 mRNA (21). Shionogi tumors, spleen, thymus, and brain were harvested 4 days postcastration for RNA extraction from mice given antisense Bcl-2 or mismatch control ODN under the same treatment schedule described above. Although Bcl-2 mRNA expression was significantly lower in tumor tissues, antisense Bcl-2 ODN had no effect on Bcl-2 expression levels in several normal organs, including spleen, thymus, and brain (Fig. 7).

DISCUSSION

Previous studies have identified a strong association between Bcl-2 and prostate cancer progression, especially with androgen-independence (4–9). For example, the introduction of bcl-2 cDNA into LNCaP prostate cancer cells increases their in vivo tumorigenic potential and resistance to apoptosis induced by androgen ablation (4), and Bcl-2 protein expression is higher in LNCaP cells that have metastasized in nude mice (5). Furthermore, increased expression of Bcl-2 in prostate cancer has been correlated with poor prognosis (6) and the emergence of AI tumors (8). Collectively, these findings suggest that the inhibition of Bcl-2 up-regulation precipitated by androgen ablation may enhance castration-induced apoptosis and delay AI progression of prostate cancer.

Antisense ODN therapy offers one strategy to specifically target bcl-2 gene expression. Phosphorothioate ODNs are water-soluble, stable agents manufactured to resist nuclease digestion. After parental administration, phosphorothioate ODNs become associated with high-capacity, low-affinity serum-binding proteins (22). Recent reports have shown that antisense Bcl-2 ODNs induce apoptosis in Bcl-2 positive small cell lung cancer cell lines in vitro (14) and increase chemosensitivity of melanoma cells in vitro and in vivo (15). Hammerhead anti-Bcl-2 ribozyme treatment of LNCaP cells reduces Bcl-2 levels and induces apoptosis in low-Bcl-2-expressing LNCaP variants in vitro (23). Taken together, these preclinical data support the hypothesis that targeting bcl-2 gene expression using antisense ODNs is a valid therapeutic strategy. The objectives of our studies were to evaluate the effects of androgen ablation on Bcl-2 expression...
INHIBITION OF SHIONOGI TUMOR GROWTH BY ANTISENSE Bcl-2 ODN

In this study, phosphorothioate antisense Bcl-2 ODN corresponding to the mouse bcl-2 translation initiation site inhibited expression of Bcl-2 mRNA and protein in a dose-dependent manner. Sequence specificity was confirmed using a 2-base Bcl-2 mismatch ODN, which had no effects on Bcl-2 mRNA and protein expression in Shionogi tumor cells. Furthermore, we demonstrated that antisense Bcl-2 ODNs decreased Bcl-2 expression in a target-specific manner; that is, the expression of other mRNAs, including related isotypes, Bax and Bcl-xL, were not affected by antisense Bcl-2 ODN treatment.

In our in vivo experiments, administration of antisense Bcl-2 ODNs accelerated castration-induced tumor regression and delayed time to AI progression compared with that of mismatch control ODN. Similar to our in vitro treatments, in vivo treatment of mice bearing Shionogi tumors with antisense Bcl-2 ODN also inhibited the Bcl-2 mRNA expression. These findings illustrate that in vivo systemic administration of ODN can result in sequence-specific down-regulation of a target gene in tumor cells. Enhanced cleavage of PARP protein in Shionogi tumors by antisense Bcl-2 ODN suggests that in vivo inhibition of Bcl-2 expression results in the earlier induction of castration-induced apoptosis in tumor tissues.

The sequence-specificity of Bcl-2 mRNA suppression observed in these in vitro and in vivo studies supports an antisense mechanism of action for the antisense ODN, although additional therapeutically beneficial, sequence-independent, nonantisense interactions cannot be ruled out (24, 25). For example, nonspecific immunostimulation by phosphorothioate ODNs can occur via natural killer-cell activation (26). Phosphorothioate ODNs have also been shown to competitively inhibit a variety of nucleases and polymerases (27, 28) and to interact with heparin-binding growth factors (29). However, nonspecific in vivo activity was not observed in our studies using phosphorothioate mismatch control ODNs. Despite distinct sequence-specific Bcl-2 suppression and significant in vivo activity, a cytotoxic effect of antisense Bcl-2 ODN was not observed in Shionogi tumor cells in vitro (data not shown). Induction of apoptosis in vitro has been reported by other investigators after treatment with antisense Bcl-2 ODN (14, 15) or ribozyme (23). This discrepancy may result from varying sensitivity to specific apoptotic stimuli in different cell lines. Furthermore, the relative balance between death antagonists and death agonists after androgen withdrawal may differ under in vitro and in vivo conditions. Androgen-regulated gene expression and growth sensitivity in AD tissues is significantly altered when transferred to in vitro monolayer culture (10, 30).

Whether antisense ODNs targeted against a specific cellular regulatory molecule have toxic effects on nondiseased organs remains undefined. Because Bcl-2 plays a critical role in some normal organs including brain and thymus (31, 32), the effects of antisense Bcl-2 ODNs on Bcl-2 expression levels in these organs were examined in DD/S mice bearing Shionogi tumors. Despite the significant decrease in Bcl-2 expression in tumor tissues, Bcl-2 expression seemed unaffected by antisense ODN in the normal organs examined. Indeed, no side effects in either antisense Bcl-2 or mismatch control ODN in Shionogi tumors after castration and during AI progression and to determine whether the combination of antisense Bcl-2 ODN and androgen withdrawal therapy could delay time to AI progression.

The Shionogi tumor model is a xenograft of an AD mouse mammary carcinoma that grows s.c. in male syngeneic hosts. Shionogi tumor cells are highly tumorigenic and locally invasive. Androgen withdrawal precipitates apoptosis and tumor regression in a highly reproducible manner. Despite complete regression after castration, rapidly growing AI Shionogi tumors invariably recur after 1 month, which provides a reliable end point to evaluate agents that can delay time to AI progression (10). We demonstrated by Northern blot analysis that Bcl-2 mRNA is up-regulated in Shionogi tumors after castration and in AI recurrent tumors. Changes in Bcl-2 expression in human prostate cancer during AI progression is similar to that in the Shionogi tumor model (8, 9).

In this study, phosphorothioate antisense Bcl-2 ODN was extracted from Shionogi tumor, spleen, thymus, and brain 4 days postcastration, and Bcl-2 and G3PDH mRNA levels were analyzed by Northern blotting.
treatment group in the present in vivo study were observed. Monia et al. reported reduced C-raf mRNA levels in mice tissues after i.v. administration of antisense C-raf mRNA ODN; however, they also observed no significant toxicities resulting from these effects (12). A Phase I dose-escalation trial using antisense Bcl-2 ODNs in nine patients with lymphoma reported objective and subjective responses with no significant toxicity (33). These findings suggest that tumor tissues may be more sensitive to phosphorothioate ODN treatment compared with normal organs, possibly because of a preferential uptake of ODN in tumor tissues for reasons of biodistribution or increased membrane permeability.

To date, new nonhormonal therapies have been traditionally evaluated in patients with hormone-refractory disease, and, when used in this end-stage setting, none has demonstrated improved survival (2). A more rational strategy to delay emergence of the AI phenotype would initiate treatment earlier to enhance castration-induced apoptosis by targeting the adaptive changes in gene expression precipitated by androgen withdrawal rather than the conventional approach of treating patients with established hormone-refractory disease. The appropriate timing of combination therapies, based on biological mechanism of progression and castration-induced changes in gene expression, may provide means to delay AI progression in a major way. The present study provides indirect evidence to further support a functional role for Bcl-2 in AI progression and demonstrates that the reduction of bcl-2 gene expression by antisense Bcl-2 ODNs inhibits progression to androgen-independence in the Shionogi tumor model. This preclinical data provides support for clinical studies with antisense Bcl-2 ODNs used adjuvantly with androgen ablation in patients with prostate cancer.

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