Synergistic antitumor effect of combined use of adenoviral-mediated p53 gene transfer and antisense oligodeoxynucleotide targeting clusterin gene in an androgen-independent human prostate cancer model

Kazuki Yamanaka,1 Martin E. Gleave,1,2 Isao Hara,3 Mototsugu Muramaki,1 and Hideaki Miyake4

1Prostate Centre, Vancouver General Hospital; 2Division of Urology, University of British Columbia, Vancouver, British Columbia, Canada; 3Department of Urology, Kobe University School of Medicine, Kobe, Japan; and 4Department of Urology, Hyogo Medical Center for Adults, Akashi, Japan

Abstract
Our recent studies showed that antisense oligodeoxynucleotide targeting antiapoptotic gene, clusterin, enhanced apoptosis induced by conventional therapeutic modalities using several prostate cancer models. In this study, to establish a more effective therapeutic strategy against prostate cancer, we investigated the effect of combined treatment with antisense clusterin oligodeoxynucleotide and adenoviral-mediated p53 gene transfer (Ad5CMV-p53) in an androgen-independent human prostate PC3 tumor model. Treatment of PC3 cells with 500 nmol/L antisense clusterin oligodeoxynucleotide decreased clusterin mRNA by >80% compared with that with 500 nmol/L mismatch control oligodeoxynucleotide. Clusterin mRNA expression in PC3 cells was highly up-regulated by Ad5CMV-p53 treatment; however, antisense clusterin oligodeoxynucleotide treatment further suppressed clusterin expression in PC3 cells after Ad5CMV-p53 treatment. Antisense clusterin oligodeoxynucleotide treatment significantly enhanced the sensitivity of Ad5CMV-p53 in a dose-dependent manner, reducing the IC50 of Ad5CMV-p53 by 75%. Apoptotic cell death was detected after combined treatment but not after treatment with either agent alone. In vivo administration of antisense clusterin oligodeoxynucleotide and Ad5CMV-p53 resulted in a significant inhibition of s.c. PC3 tumor growth as well as lymph node metastases from orthotopic PC3 tumors compared with administration of either agent alone. Furthermore, combined treatment with antisense clusterin oligodeoxynucleotide, Ad5CMV-p53, and mitoanxantrone completely eradicated s.c. PC3 tumors and lymph node metastases from orthotopic PC3 tumors in 60% and 100% of mice, respectively. These findings suggest that combined treatment with antisense clusterin oligodeoxynucleotide and Ad5CMV-p53 could be a novel strategy to inhibit progression of hormone-refractory prostate cancer and that further addition of chemotherapeutic agents may help to enhance the efficacy of this combined regimen.

Introduction
Prostate cancer is the most commonly diagnosed malignant disease and the second leading cause of cancer-related death in men in Western industrialized countries. Androgen ablation, however, remains the only effective therapy for patients with advanced disease. Approximately 80% of patients achieve symptomatic and/or objective response after androgen withdrawal, but progression to androgen independence ultimately occurs in almost all cases (1). Although a number of nonhormonal agents have been evaluated in patients with hormone-refractory disease, these agents have limited antitumor activity with an objective response rate of <20% and failed to show a survival benefit (2). Accordingly, novel therapeutic strategies targeting molecular mechanisms mediating resistance to conventional treatments must be developed to achieve a significant impact on the survival of patients with advanced prostate cancer.

Gene transfer techniques may provide new therapeutic approaches to a variety of malignancies (3), and among them, replication-deficient adenoviral vectors have been shown to be attractive because they are capable of efficient infection of target cells and are safe for clinical use (4). A number of studies have shown that adenoviral-mediated gene therapy of wild-type p53 in tumor cells, including prostate cancer, suppressed tumor growth both in vitro and in vivo through induction of the apoptotic pathway. Based on the promising results of these preclinical studies, several clinical trials of p53 gene therapy have been carried out (5–10). Recent studies, however, showed that expression of antiapoptotic genes markedly inhibits the therapeutic efficacy of adenoviral-mediated p53 gene transfer (11, 12);
therefore, these findings suggest that it would be necessary to add another type of adequate therapy if adenovirus \( p53 \) vectors are used for cases expressing high levels of antiapoptotic genes.

Clusterin, also known as testosterone-repressed prostate message-2, sulfated glycoprotein-2, or apolipoprotein J, was first isolated from ram rete testes fluid (13) and has various biological functions, including tissue remodeling, reproduction, lipid transport, and apoptotic cell death (14). Clusterin was initially regarded as a marker for cell death, because its expression is highly up-regulated in various normal and malignant tissues undergoing apoptosis (15–18). Recent studies, however, reported conflicting findings on the association between enhanced clusterin expression and apoptotic activity (19–21). Similarly, clusterin expression is increased in regressing normal and malignant prostate tissues after androgen ablation (15), and its up-regulation has been shown to be associated with antiapoptotic activity and disease progression in prostate cancer (22–24). We have also reported that clusterin expression in malignant prostatic tissue was significantly greater in patients who underwent preoperative neoadjuvant hormonal therapy (25), and that clusterin expression in prostate cancer cells plays a protective role against apoptosis induced by androgen ablation, cytotoxic chemotherapeutic agent, has been shown to induce more potent antineoplastic effects in some tumor model systems (26–28). However, because numerous genes mediate tumor progression, inhibition of a single target gene is likely insufficient to completely suppress tumor progression. Although there has been no report demonstrating complete responses of established tumors in vivo using antisense oligodeoxynucleotide technique alone, combined use of antisense oligodeoxynucleotide with another oligodeoxynucleotide targeting a different gene or other type of agents, such as a chemotherapeutic agent, has been shown to induce more potent antineoplastic effects in some tumor model systems (29, 30).

The objectives of this study were to test whether induction of apoptotic cell death by adenoviral-mediated \( p53 \) gene transfer (Ad5CMV-\( p53 \)) is enhanced by antisense clusterin oligodeoxynucleotide treatment, and to determine whether combined use of Ad5CMV-\( p53 \) and antisense clusterin oligodeoxynucleotide inhibits tumor growth and metastasis in the androgen-independent human prostate PC3 tumor model.

Materials and Methods

Tumor Cell Line

PC3, derived from human prostate cancer, was purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% heat-inactivated FCS.

Chemotherapeutic Agent

Mitoxantrone was purchased from Wyeth-Ayerst, Inc. (Montreal, Quebec, Canada). Stock solutions of mitoxantrone (1 mg/mL) were prepared with DMSO and diluted with PBS to the required concentrations before each in vitro experiment.

Adenovirus Vectors

Two previously described replication-deficient recombinant adenovirus vectors were used in this study (12). Ad5CMV-\( p53 \) expresses wild-type human \( p53 \) under the control of the human cytomegalovirus promoter, and Ad5CMV-Luc encoding Luciferase gene was used as a control vector. Adenoviruses were propagated in 293 cells, a human embryonic kidney cell line, which contains estrone and is thus highly permissive of the replication of the estrone replication-deficient adenovirus. It was stored as previously reported, and the viral infection titers were determined by plaque assays (12).

Antisense Clusterin Oligodeoxynucleotides

Phosphorothioate oligodeoxynucleotides used in this study were generously supplied by Dr. Brett P. Monia (Isis Pharmaceuticals, Carlsbad, CA). The sequences of antisense clusterin oligodeoxynucleotides corresponding to the human clusterin translation initiation site were 5′-CAGCAGACAGCAGCTCAT-3′. Two-base mismatch antisense oligodeoxynucleotide (5′-CAGCAGACTTTATAT-3′) was used as control. Oligodeoxynucleotides were diluted in 10 mmol/L Tris (pH 7.4) and 1 mmol/L EDTA and kept at −20°C.

Treatment of Cells with Oligodeoxynucleotides

PC3 cells were treated with various concentrations of oligodeoxynucleotides after a preincubation for 20 minutes with 4 μg/mL lipofectin (Life Technologies) in serum-free OPTI-MEM (Life Technologies). Four hours later, the medium containing oligodeoxynucleotides and lipofectin was replaced with the standard culture medium described above.

Northern Blot Analysis

Total RNA was isolated from cultured PC3 cells using the acid-guanidium thiocyanate-phenol-chloroform method. Electrophoresis, hybridization, and washing conditions were carried out as previously reported (28). Human clusterin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were generated by reverse transcription-PCR from total RNA of human kidney using primers 5′-AAGGAAATTCA-AAATGCTGTAAA-3′ (sense) and 5′-ACAGACAGACCTCCCAGCAGC-3′ (antisense) for clusterin and 5′-TGCTTTTAACTCCGTTTTCTAG-3′ (antisense) for GAPDH. The density of bands for clusterin was normalized against that of GAPDH by densitometric analysis.

Real-time Reverse Transcription-PCR

Total RNA was isolated as described above, and 1 μg of each total RNA was reverse transcribed using an Oligo dT and Superscript premiﬂation system (Life Technologies). To examine the expression levels of clusterin, real-time quantitative PCR was done using a Sequence Detector (ABI PRISM 7700, PE Applied Biosystems, Foster City, CA).
Selected sequences of primers, and probes are as follows: 5'-GAGCAGCTGAAAGCAGCTT-3' (sense), 5'-CTTCCCGCTGAGGT-3' (antisense) and 5'-ACTGCTTCTCC-CGGCT-3' (probe) for clusterin and 5'-GAAGGTAAGGCTCGACT-3' (sense), GAPDH 5'-GAAGATGTTGATGGGATTTC-3' (antisense), 5'-CAAGCTTCGGTTCTCACGCC-3' (probe) for GAPDH. The Taqman probes consisted of an oligodeoxynucleotide with a 5' FAM reporter dye and 3' TAMRA quencher dye. Each cDNA was analyzed by quantitative PCR in a 50-μL volume using Master Mix (PE Applied Biosystems). The condition of thermal cycling was 50 cycles of amplification consisting of 15 seconds at 95°C and 1 minute at 60°C. Real-time quantitation was done based on Taqman assay according to the manufacturer's instruction. After the generation of a real-time amplification plot based on the normalized fluorescence signal, the threshold cycle was determined. Threshold cycle was then used for kinetic analysis and was proportional to the initial number of target copies in the sample. The starting quantity of a sample was calculated after comparison of the threshold cycles of a serial dilution of a positive control. Transcripts of the GAPDH were also quantified as an internal reference, and the quantification value of clusterin mRNA was described as each value relative to that of GAPDH mRNA.

**In vitro Cell Growth Assay**

The in vitro growth-inhibitory effects of antisense clusterin oligodeoxynucleotide and/or Ad5CMV-p53 on PC3 cells were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) as described previously (28). Briefly, 1 × 10² cells were seeded in each well of 96-well microtiter plates and allowed to attach overnight. Cells were then treated once daily with various concentrations of oligodeoxynucleotide for 2 days. Following oligodeoxynucleotide treatment, cells were treated with various concentrations of Ad5CMV-p53. After 48 hours of incubation, 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in PBS was added to each well followed by incubation for 4 hours at 37°C. The formazan crystals were dissolved in DMSO. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware, Lincoln Park, NJ) at 540 nm. Absorbance values were normalized to the values obtained (Becton Dickinson Labware, Lincoln Park, NJ) at 540 nm. Absorbance values were normalized to the values obtained (Becton Dickinson Labware, Lincoln Park, NJ) at 540 nm.

**Measurement of Apoptosis**

The nucleosomal DNA degradation was quantified by the Cell Death Detection ELISA kit using antihistone antibody (Roche, Mannheim, Germany) as described previously (31). Briefly, 1 × 10² PC3 cells were seeded in 5-cm culture dishes and allowed to adhere overnight. After treatment with oligodeoxynucleotide and/or Ad5CMV-p53 under the same schedule as described above, cells were harvested and then assays were done in triplicate according to the manufacturer's instruction.

**Assessment of In vivo Tumor Growth**

Athymic nude mice (BALB/c nu/nu females, 6–8 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan), and housed in a controlled environment at 22°C on a 12-hour light, 12-hour dark cycle. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Each experimental group consisted of 10 mice. PC3 cells were trypsinized, washed twice with PBS, and 1 × 10⁵ cells were injected s.c. with 0.1 mL of Matrigel (Becton Dickinson Labware) in the flank, or directly given 1 × 10⁶ cells into the prostate, as previously described (32). The schedules administrating oligodeoxynucleotide, viral vectors, and chemotherapeutic agents were determined based on our previously reported studies (8, 28, 33).

For the first experiment, 10 days after injection, mice were randomly selected for treatment with antisense clusterin oligodeoxynucleotide plus Ad5CMV-Luc, mismatch control oligodeoxynucleotide plus Ad5CMV-Luc, antisense clusterin oligodeoxynucleotide plus Ad5CMV-p53, or mismatch control oligodeoxynucleotide plus Ad5CMV-p53. After randomization, 10 mg/kg antisense clusterin or mismatch control oligodeoxynucleotide were injected i.p. once daily into each mouse for 28 days, and 200 μL of adenovirus vector [1 × 10⁹ plaque-forming units (pfu)/mL] were injected intratumorally or i.p. into mice bearing s.c. or orthotopic tumors, respectively, twice a week for 2 weeks.

For the second experiment, 10 days after injection, mice were randomly selected for treatment with antisense clusterin oligodeoxynucleotide, Ad5CMV-Luc plus mitoxantrone, mismatch control oligodeoxynucleotide, Ad5CMV-Luc plus mitoxantrone, antisense clusterin oligodeoxynucleotide, Ad5CMV-p53 plus mitoxantrone, or mismatch control oligodeoxynucleotide, Ad5CMV-p53 plus mitoxantrone. After randomization, 10 mg/kg antisense clusterin or mismatch control oligodeoxynucleotide was injected i.p. once daily into each mouse for 28 days, 200 μL of adenovirus vector (1 × 10⁹ plaque-forming units (pfu)/mL) were injected intratumorally or i.p. into mice bearing s.c. or orthotopic tumors, respectively, twice a week for 2 weeks, and 50 μg of mitoxantrone were injected i.v. twice a week for 2 weeks.

Tumor volume was measured every 5 days and calculated by the formula length × width × depth 0.5236 (28). Eight weeks after the injection of tumor cells in the prostate, the mice were sacrificed and the presence of metastasis was macroscopically examined in all abdominal and thoracic internal organs. The organs with metastases were removed, and the number of surface metastatic nodules was counted.

**Statistical Analysis**

A repeated-measure ANOVA model was used to analyze the both in vitro and in vivo cytotoxic effects of oligodeoxynucleotide, Ad5CMV-p53 and mitoxantrone. Changes in clusterin mRNA levels were evaluated by one-factor ANOVA. Synergy of oligodeoxynucleotide and Ad5CMV-p53 was analyzed by calculating the fractional product variable according to the fractional product method. Other
data were analyzed by Student’s *t* test. The levels of statistical significance were set at *P* < 0.05, and all statistical calculations were done using Statview 4.5 software (Abacus Concepts, Inc., Berkeley, CA).

**Results**

Changes in Clusterin Expression in PC3 Cells after Treatment with Ad5CMV-p53 and/or Antisense Clusterin Oligodeoxynucleotides

We initially showed the marked inhibition of clusterin mRNA expression in PC3 cells by treatment with antisense clusterin oligodeoxynucleotide using Northern blot analysis; that is, treatment of PC3 cells with 500 nmol/L antisense clusterin oligodeoxynucleotide decreased clusterin mRNA by >80% compared with that with mismatch control oligodeoxynucleotide (Fig. 1A).

Real-time reverse transcription-PCR assay was used to determine the effects of Ad5CMV-p53 treatment on clusterin mRNA expression in PC3 cells. As shown in Fig. 1B, clusterin mRNA induction increased in a dose-dependent manner by Ad5CMV-p53 treatment at concentrations up to 50 pfu per cell (*P* < 0.05). Time course experiment showed that Ad5CMV-p53-induced clusterin mRNA up-regulation peaked by 96 hours after treatment and began decreasing by 120 hours after treatment (*P* < 0.05; Fig. 1C).

We then examined whether antisense clusterin oligodeoxynucleotide treatment further suppresses clusterin expression in PC3 cells treated by Ad5CMV-p53, which causes up-regulation of the clusterin gene as described above. As shown in Fig. 1D, combined treatment with 500 nmol/L antisense clusterin oligodeoxynucleotide and 5 or 10 pfu per cell Ad5CMV-p53 decreased clusterin mRNA levels by 84% or 87%, respectively, compared with that by 500 nmol/L mismatch control oligodeoxynucleotide treatment and 5 or 10 pfu per cell Ad5CMV-Luc.

**Figure 1.** Effects of antisense (AS) clusterin oligodeoxynucleotide (ODN) and/or Ad5CMV-p53 treatment on clusterin expression in PC3 cells. **A**, PC3 cells were treated daily with antisense clusterin oligodeoxynucleotide or a two-base clusterin mismatch (MM) control ODN for 2 d. Total RNA was extracted from cultured cells and analyzed for clusterin and GAPDH levels by Northern blotting. No Tx, untreated cells. **B**, PC3 cells were treated with various doses of Ad5CMV-p53 for 48 h; total RNA was then extracted and analyzed for clusterin levels by real-time reverse transcription-PCR. As described in Materials and Methods, clusterin mRNA value was corrected by corresponding GAPDH mRNA value. Columns, mean of three independent experiments; bars, SD. The changes in clusterin mRNA levels were significant (*P* < 0.05 by one-factor ANOVA). **C**, PC3 cells were treated with Ad5CMV-p53 at 10 pfu per cell for indicated intervals; total RNA was then extracted and analyzed for clusterin levels by real-time reverse transcription-PCR. As described in Materials and Methods, clusterin mRNA value was corrected by corresponding GAPDH mRNA value. Columns, mean of three independent experiments; bars, SD. The changes in clusterin mRNA levels were significant (*P* < 0.05 by one-factor ANOVA). **D**, PC3 cells were treated daily with 500 nmol/L antisense clusterin ODN or a two-base clusterin mismatch (MM) control ODN for 2 d. Following a 48-h exposure to Ad5CMV-p53 at 5 or 10 pfu per cell, total RNA was extracted and clusterin levels were analyzed by real-time reverse transcription-PCR. As described in Materials and Methods, clusterin mRNA value was corrected by corresponding GAPDH mRNA value. Columns, mean of three independent experiments; bars, SD. *, *P* < 0.01, differs from control (Student’s *t* test).
Synergistic Induction of Apoptotic Cell Death by Ad5CMV-p53 and Antisense Clusterin Oligodeoxynucleotides in PC3 Cells In vitro

To examine whether treatment with antisense clusterin oligodeoxynucleotide enhances the Ad5CMV-p53-induced cytotoxicity, PC3 cells were treated with various concentrations of antisense clusterin oligodeoxynucleotide once daily for 2 days and then incubated with various concentrations of Ad5CMV-p53 for 2 days. As shown in Fig. 2A, antisense clusterin oligodeoxynucleotide treatment significantly enhanced sensitivity to Ad5CMV-p53 in a dose-dependent manner (P < 0.01), reducing the IC50 of Ad5CMV-p53 from 25 to 5 pfu per cell, whereas mismatch control oligodeoxynucleotide had no effect. Dose-dependent synergy between antisense clusterin oligodeoxynucleotide and Ad5CMV-p53 was also observed by increasing the antisense oligodeoxynucleotide concentration when Ad5CMV-p53 dose was fixed at 5 pfu per cell (P < 0.01; Fig. 2B).

DNA fragmentation was measured using an ELISA kit to evaluate the effects of combined antisense clusterin oligodeoxynucleotide (500 nmol/L) and Ad5CMV-p53 (5 pfu per cell) treatment on apoptosis induction. After the same treatment schedule described above, marked DNA ladder formation was observed only after combined treatment with antisense clusterin oligodeoxynucleotide and Ad5CMV-p53 (Fig. 2C).

Inhibition of PC3 Tumor Growth and Metastasis In vivo by Combined Treatment with Ad5CMV-p53 and Antisense Clusterin Oligodeoxynucleotides

The efficacy of a regimen combining Ad5CMV-p53 and antisense clusterin oligodeoxynucleotide for inhibiting the growth of s.c. PC3 tumors was evaluated. Athymic nude mice bearing PC3 tumors ~1 cm in diameter were randomly selected for treatment as described above. Mean tumor volume was similar at the beginning of treatment in each of these groups. Whereas changes in tumor volume in mice treated with antisense clusterin oligodeoxynucleotide plus Ad5CMV-Luc were similar to those with mismatch control oligodeoxynucleotide plus Ad5CMV-Luc, PC3 tumor growth was inhibited slightly but definitively by treatment with mismatch control oligodeoxynucleotide plus Ad5CMV-p53, and combined antisense clusterin oligodeoxynucleotide and Ad5CMV-p53 therapy showed marked growth inhibitory effects. Fifty days after tumor injection, the tumor volume in mice treated with antisense clusterin oligodeoxynucleotide plus Ad5CMV-p53 was 65%, 66%, or 50% smaller than that in mice treated with antisense clusterin oligodeoxynucleotide plus Ad5CMV-Luc, mismatch control oligodeoxynucleotide plus Ad5CMV-Luc, or mismatch control oligodeoxynucleotide plus Ad5CMV-p53, respectively (Fig. 3); however, there were no significant differences in the body weight of mice among these four groups (data not shown).

We then examined the effects of the combined regimen using a recently reported orthotopic injection (i.e., intraprostatic injection) model (32) according to the same treatment schedule described above. As shown in Table 1, the
The incidence of metastasis or hemorrhagic ascites was significantly different from that in mice treated with other regimens (Ad5CMV-p53, antisense clusterin oligodeoxynucleotide plus Ad5CMV-Luc, or mismatch control oligodeoxynucleotide plus Ad5CMV-p53). In addition, when sacrificed, there were no significant differences in the body weight of mice among these four groups (data not shown).

Complete Eradication of PC3 Tumor In vivo by Combined Treatment with Ad5CMV-p53, Antisense Clusterin Oligodeoxynucleotide, and Mitoxantrone

To achieve more potent antitumor effect against PC3 tumor growth, we evaluated the efficacy of combined regimen consisting of Ad5CMV-p53, antisense clusterin oligodeoxynucleotide, and mitoxantrone. Athymic nude mice bearing s.c. PC3 tumors ~1 cm in diameter were randomly selected for treatment as described above. Mean tumor volume was similar at the beginning of treatment in each of these groups. As shown in Fig. 4, combined treatment with Ad5CMV-p53, antisense clusterin oligodeoxynucleotide, and mitoxantrone significantly inhibited PC3 tumor growth compared with the other three regimen, and PC3 tumors in 6 of 10 mice were completely eradicated by this combined therapy. Furthermore, combined treatment with Ad5CMV-p53, antisense clusterin oligodeoxynucleotide, and mitoxantrone inhibited the development of lymph node metastasis in all mice after PC3 cells were orthotopically injected (Table 2). However, there were no significant differences in the body weight of mice among these four groups in s.c as well as orthotopic tumor cell injection models (data not shown).

Discussion

Despite the original hypothesis that clusterin is a marker for programmed cell death (15–18), several studies showed conflicting findings suggesting the dissociation of up-regulation of clusterin gene from apoptosis (19–21, 23–25). We also previously showed that overexpression of clusterin helps mediate malignant progression against apoptosis induced by therapeutic stimuli using several kinds of preclinical animal models, including prostate cancer (26–28). Collectively, these findings indicate that

combined antisense clusterin oligodeoxynucleotide and Ad5CMV-p53 therapy substantially suppressed the primary tumor growth as well as the incidence of metastasis after orthotopic injection of PC3 cells compared with the other three treatment regimens. That is, both the weight of primary PC3 tumor and the incidences of retroperitoneal lymph node metastasis in mice treated with combined antisense clusterin oligodeoxynucleotide and Ad5CMV-p53 were significantly lower than those in mice treated with antisense clusterin oligodeoxynucleotide plus Ad5CMV-Luc, mismatch control oligodeoxynucleotide

Table 1. Changes in metastasis of PC3 cells injected into the prostate of nude mice after treatment with antisense clusterin oligodeoxynucleotide and Ad5CMV-p53

<table>
<thead>
<tr>
<th>Regimen*</th>
<th>Incidence of lymph node metastasis (%)†</th>
<th>Incidence of hemorrhagic ascites (%)‡</th>
<th>Weight of the primary tumor (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS clusterin ODN + Ad5CMV-Luc</td>
<td>10/10 (100)</td>
<td>4/10 (40)</td>
<td>24.2 ± 8.3‡</td>
</tr>
<tr>
<td>MM control ODN + Ad5CMV-Luc</td>
<td>10/10 (100)</td>
<td>4/10 (40)</td>
<td>26.1 ± 10.1</td>
</tr>
<tr>
<td>AS clusterin ODN + Ad5CMV-p53</td>
<td>3/10 (30)</td>
<td>0/10 (0)</td>
<td>13.6 ± 5.1‡</td>
</tr>
<tr>
<td>MM control ODN + Ad5CMV-p53</td>
<td>7/10 (70)</td>
<td>2/10 (20)</td>
<td>19.6 ± 7.2</td>
</tr>
</tbody>
</table>

*Ten days after the implantation of tumor cells, 10 mg/kg antisense clusterin oligodeoxynucleotide (AS clusterin ODN) or mismatch control oligodeoxynucleotide (MM control ODN) was injected i.p. for 28 days, and 200 µL of adenoviral vector (1 × 10⁷ pfu/mL) was injected i.t. twice a week for 2 wks.
†No. with tumor/no. injected mice.
‡No. with hemorrhagic ascites/no. injected mice.
§Mean ± SD.
∥The incidence of metastasis or hemorrhagic ascites was significantly different from that in mice treated with other regimens (P < 0.05, Student’s t test).
*The mean weight of the primary tumor was significantly different from that in mice treated with other regimens (P < 0.05, Student’s t test).
clusten expression plays a protective role against apoptosis induced by various kinds of stimuli, and thereby may confer an aggressive phenotype during cancer progression.

Advances in the field of nucleic acid chemistry offers one attractive strategy to design antisense oligodeoxynucleotide-based therapeutic agents, that specifically hybridize with complementary mRNA regions of a target gene and thereby inhibit gene expression by forming RNA/DNA duplexes (36). Rapid intracellular degradation of oligodeoxynucleotide is a potential disadvantage of antisense oligodeoxynucleotide therapy, but this problem can be overcome by substituting a nonbridging phosphoryl oxygen of DNA with a sulfur to create a phosphorothioate backbone, which stabilizes the oligodeoxynucleotide against nuclease digestion (37, 38). Recently, several antisense oligodeoxynucleotides targeted against specific genes involved in neoplastic progression have been evaluated as potential therapeutic agents (29–33, 35, 38–42). We also previously showed that antisense clusterin oligodeoxynucleotide used in this study enhanced the therapeutic effects of androgen ablation and cytotoxic chemotherapy in several kinds of prostate cancer xenograft models (26–28). These findings clarify the efficacy of antisense oligodeoxynucleotide as a novel class of antineoplastic agents when designed against appropriate molecular targets. However, because numerous genes are involved in tumor progression, inhibition of a single target gene may likely be insufficient to completely inhibit tumor growth.

Recently, the usefulness of p53 tumor suppressor gene therapy has been reported as a possible new therapeutic approach against various kinds of malignant diseases, including prostate cancer (5–10). Among several kinds of vectors, a replication-deficient adenoviral vector encoding human wild-type p53 under the control of a human cytomegalovirus promoter (i.e., Ad5CMV-p53) has been most frequently used because of various useful properties including a high-infectious ability, limited period of the transgene expression, and very low probability of integration of the transgene into the host genome (4). However, the molecular mechanism interfering with the effects of the p53 gene therapy has been clarified; that is, if antiapoptotic genes, such as bcl-2, were overexpressed in target malignant lesions, the efficacy of adenoviral-mediated p53 gene transfer would be significantly limited through the inhibition of apoptotic cell death (31, 32). In this study, therefore,

Table 2. Changes in metastasis of PC3 cells injected into the prostate of nude mice after treatment with antisense clusterin oligodeoxynucleotide, Ad5CMV-p53, and mitoxantrone

<table>
<thead>
<tr>
<th>Regimen*</th>
<th>Incidence of lymph node metastasis (%)</th>
<th>Incidence of hemorrhagic ascites (%)</th>
<th>Weight of the primary tumor (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS clusterin ODN + Ad5CMV-Luc + mitoxantrone</td>
<td>8/10 (80)</td>
<td>2/10 (20)</td>
<td>20.2 ± 6.9*</td>
</tr>
<tr>
<td>AS control ODN + Ad5CMV-Luc + mitoxantrone</td>
<td>9/10 (90)</td>
<td>2/10 (20)</td>
<td>24.1 ± 9.1</td>
</tr>
<tr>
<td>AS clusterin ODN + Ad5CMV-p53 + mitoxantrone</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
<td>7.6 ± 4.8</td>
</tr>
<tr>
<td>MM control ODN + Ad5CMV-p53 + mitoxantrone</td>
<td>6/10 (60)</td>
<td>1/10 (10)</td>
<td>17.6 ± 6.1</td>
</tr>
</tbody>
</table>

*Ten days after the implantation of tumor cells, 10 mg/kg antisense clusterin oligodeoxynucleotide (AS clusterin ODN) or mismatch control oligodeoxynucleotide (MM control ODN) were injected i.p. for 28 days, 200 μL of adenoviral vector (1 × 10^7 pfu/mL) and 50 μg of mitoxantrone were injected i.p. and i.v., respectively, twice a week for 2 weeks.

†No. mice with tumor/no. injected mice.

‡No. mice with hemorrhagic ascites/no. injected mice.

§Mean ± SD.

The incidence of metastasis or hemorrhagic ascites was significantly different from that in mice treated with other regimens (p < 0.05, χ² test).

The mean weight of the primary tumor was significantly different from that in mice treated with other regimens (p < 0.05, Student’s t test).
we evaluated the efficacy of combined treatment with antisense clusterin oligodeoxynucleotide and Ad5CMV-p53 using the androgen-independent human prostate PC3 tumor model.

We initially evaluated the effect of Ad5CMV-p53 treatment on clusterin expression level in PC3 cells, because clusterin expression has been shown to be highly up-regulated in various tissues undergoing apoptotic cell death (15–18, 26–28). As expected, clusterin expression in PC3 cells was markedly increased after Ad5CMV-p53 treatment, suggesting that clusterin up-regulation is likely an adaptive response, which helps the cell survival against p53-dependent cell death signal. However, phosphorothioate antisense clusterin oligodeoxynucleotide inhibited clusterin mRNA in dose-dependent and sequence-specific manners, even after Ad5CMV-p53 treatment, which resulted in a significant increase in clusterin expression. Furthermore, treatment of PC3 cells with antisense clusterin oligodeoxynucleotide reduced the IC50 of Ad5CMV-p53 by 75% and enhanced Ad5CMV-p53-induced apoptosis, although no growth inhibitory effects were observed in PC3 cells treated with antisense clusterin oligodeoxynucleotide alone. These findings suggest that clusterin expression in androgen-independent prostate cancer cells may confer a phenotype resistant to apoptosis induced by Ad5CMV-p53; therefore, despite the lack of a significant effect on cell proliferation in the absence of other apoptotic stimuli or cell death signals, the inhibition of clusterin expression by antisense clusterin oligodeoxynucleotide may enhance the sensitivity to several therapies inducing p53-dependent apoptosis for androgen-independent prostate cancer.

Recently, several investigators have shown that over-expression of antiapoptotic genes in prostate cancer cells, such as mutant-type p53, bcl-2, and clusterin, helps mediate resistance to conventional treatment through the inhibition of apoptotic cell death (23, 43, 44). These findings suggest that the approach of enhancing sensitivity to stimuli, which induces apoptotic cell death, by decreasing the expression of the antiapoptotic gene seems to be a more rational strategy for patients with advanced androgen-independent prostate cancer than the conventional approach of combining several kinds of treatments. Furthermore, recent preclinical studies have provided proof of principle evidence that targeting antiapoptotic genes using antisense oligodeoxynucleotide enhances apoptosis induced by conventional therapeutic options (26–28, 30, 35). Accordingly, based on the findings of the present in vitro experiments, we then examined whether antisense clusterin oligodeoxynucleotide therapy enhances the cytotoxic effect of Ad5CMV-p53 on the growth and metastasis of PC3 cells in vivo, and showed that a regimen combining antisense clusterin oligodeoxynucleotide and Ad5CMV-p53 markedly inhibited the growth of s.c. PC3 tumors in vivo. These findings suggest that it might be possible to achieve powerful cytotoxic effects of Ad5CMV-p53 at tolerable doses by combining with antisense clusterin oligodeoxynucleotide. The combined regimen also significantly suppressed the incidence of metastasis after orthotopic injection of PC3 cells, resulting in a significant delay of tumor progression. This combined regimen may directly affect the metastatic process and also suppresses metastasis through inhibition of the growth of the orthotopically inoculated primary tumors. However, it may be potentially important for the clinical application of this combined regimen to develop the specific targeting delivery of viral vectors to the prostate as well as the stable oligodeoxynucleotide after in vivo administration.

We further evaluated the effect of combined treatment with antisense clusterin oligodeoxynucleotide, Ad5CMV-p53, and mitoxantrone on PC3 tumor growth in vivo, since mitoxantrone has been approved by the U.S. Food and Drug for use in hormone-refractory prostate cancer based on two randomized controlled trials demonstrating improved palliative response rates (45, 46). Surprisingly, the combined therapy with these three agents eradicated established PC3 tumor from 6 of 10 mice. There were no serious side effects were observed by the administration of antisense clusterin oligodeoxynucleotide and Ad5CMV-p53, and the dose of mitoxantrone used in this experiment was tolerable; therefore, this combined treatment could be done safely and provide significant antitumor effects.

In conclusion, the present findings suggest that expression of clusterin inhibit the effect of Ad5CMV-p53 treatment (i.e., p53-dependent apoptosis), resulting in androgen-independent prostate cancer progression, and that decreasing clusterin expression with the use of antisense oligodeoxynucleotide targeting clusterin gene may provide a feasible and safe strategy to enhance the therapeutic efficacy of Ad5CMV-p53 in prostate cancer. The preclinical data shown here provides preliminary evidence supporting the design of clinical studies using a combination of antisense clusterin oligodeoxynucleotide plus Ad5CMV-p53 therapy for patients with advanced androgen-independent prostate cancer.

References

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Synergistic antitumor effect of combined use of adenoviral-mediated p53 gene transfer and antisense oligodeoxynucleotide targeting clusterin gene in an androgen-independent human prostate cancer model

Kazuki Yamanaka, Martin E. Gleave, Isao Hara, et al.


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