Synergistic Chemosensitization and Inhibition of Tumor Growth and Metastasis by the Antisense Oligodeoxynucleotide Targeting Clusterin Gene in a Human Bladder Cancer Model

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ABSTRACT
Clusterin expression is highly up-regulated in several normal and malignant tissues undergoing apoptosis. Although recent studies have demonstrated a protective role of clusterin expression against various kinds of apoptotic stimuli, the functional role of clusterin in the acquisition of a therapy-resistant phenotype in bladder cancer remains unknown. The objectives of this study were to determine whether antisense (AS) oligodeoxynucleotide (ODN) targeting the clusterin gene enhances apoptosis induced by cisplatin and to evaluate the usefulness of combined treatment with AS clusterin ODN and cisplatin in the inhibition of KoTCC-1 tumor growth and metastasis in a human bladder cancer KoTCC-1 model. We initially revealed the dose-dependent and sequence-specific inhibition of clusterin expression by AS clusterin ODN treatment in KoTCC-1 cells at both mRNA and protein levels. Clusterin mRNA was increased in a dose-dependent manner by cisplatin treatment at concentrations ≤10 mg/ml, and clusterin mRNA up-regulation induced by 10 mg/ml cisplatin peaked by 48-h post-treatment and began decreasing by 72-h post-treatment. Although there was no significant effect on growth of KoTCC-1 cells, AS clusterin ODN treatment significantly enhanced cisplatin chemosensitivity of KoTCC-1 cells in a dose-dependent manner, reducing the IC_{50} by >50%. Characteristic apoptotic DNA ladder formation and cleavage of poly(ADP-ribose) polymerase protein were detected after combined treatment with AS clusterin ODN and cisplatin but not either agent alone. In vivo systemic administration of AS clusterin and cisplatin significantly decreased the s.c. KoTCC-1 tumor volume compared with mismatch control ODN plus cisplatin. Furthermore, after the orthotopic implantation of KoTCC-1 cells, combined treatment with AS clusterin and cisplatin significantly inhibited the growth of primary KoTCC-1 tumors, as well as the incidence of lymph node metastasis. Collectively, these findings demonstrated that clusterin helps confer a chemoresistant phenotype through inhibition of apoptosis and that combined AS clusterin ODN may be useful in enhancing the effects of cytotoxic chemotherapy in patients with bladder cancer.

INTRODUCTION
Bladder cancer is the second most common malignancy of the genitourinary tract and the fourth or fifth leading cause of cancer-related deaths of men in Western industrialized countries. The prognosis of patients with invasive and/or metastatic bladder cancer is still extremely poor despite recent therapeutic advances (1). Because cisplatin, a coordination complex of platinum, was introduced, cisplatin-based combination chemotherapy has been the mainstay of treatment for patients with advanced bladder cancer, and it is at least palliatively effective. However, the efficacy of cisplatin-based combination chemotherapy is limited because of de novo drug resistance or the development of the cellular-resistant phenotype during treatment; therefore, there has been no study demonstrating the significant prognostic benefit of this therapy (2). It is, thus, important to clarify the factors that inhibit the effects of chemotherapeutic agents, develop novel treatments, and consequently improve the survival of patients with advanced bladder cancer.

Clusterin, also known as testosterone-repressed prostate message-2 or sulfated glycoprotein-2, was first isolated from ram rete testes fluid and plays important roles in various physiological processes, including tissue remodeling, reproduction, lipid transport, complement regulation, and apoptosis (3). Because clusterin expression is increased in various benign and malignant tissues undergoing apoptosis, it has been regarded as a marker for cell death (4, 5). Recent studies, however, have provided conflicting evidence regarding the relationship between clusterin up-regulation and increased apoptotic activity (6, 7). We also demonstrated the powerful antiapoptotic activity of clusterin using several kinds of prostate cancer models; that is, increased clusterin expression after androgen ablation accelerates tumor progression by inhibiting castration- and chemotherapy-induced apoptosis (8–11). Although our recent studies showed that overexpression of clusterin is closely associated with disease progression and recurrence in patients with bladder cancer (12), to our knowledge, there has been no study characterizing the functional significance of clusterin expression in bladder cancer.
To date, several preclinical studies have reported encouraging findings using novel agents targeting bladder cancer (13–18). Among them, advances in the field of nucleic acid chemistry offers one attractive strategy to design AS ODN-based therapeutic agents that specifically hybridize with complementary mRNA regions of a target gene and thereby inhibit gene expression by forming RNA/DNA duplexes (19). Recently, several AS ODNs targeted against specific genes involved in neoplastic progression have been evaluated as potential therapeutic agents (20–23). Collectively, these findings identify AS ODNs as a novel class of antineoplastic agents when designed for appropriate molecular targets. However, because numerous genes are involved in tumor progression, inhibition of a single target gene will likely be insufficient to inhibit tumor progression in a meaningful way (11). In fact, combined use of AS ODNs with other compounds, such as chemotherapeutic agents, has been demonstrated to produce more potent antineoplastic effects in some tumor model systems (9, 10, 24–26).

In this study, we tested whether AS ODN, targeted against the clusterin gene, enhances cisplatin chemosensitivity in human bladder cancer KoTCC-1 cells and whether combined AS clusterin ODN and cisplatin treatment cooperatively inhibit growth and metastasis of KoTCC-1 tumors using s.c. and orthotopic tumor cell injection models.

**MATERIALS AND METHODS**

**Tumor Cell Line.** The human bladder cancer cell line KoTCC-1 was established in our laboratory (15) and was maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% FCS.

**Chemotherapeutic Agent.** Cisplatin, a generous gift from Nippon Kayaku Co. (Tokyo, Japan), was dissolved in PBS at 1 mg/ml and diluted in medium before each experiment.

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Fig. 1  Sequence-specific and dose-dependent inhibition of clusterin expression by AS clusterin ODN in KoTCC-1 cells. In A, KoTCC-1 cells were treated daily with various concentrations of AS clusterin ODN (CAGCAGCAGGCTTCATCAT) or a 2-base clusterin MM ODN (CAGCAGCAGTATTTATCAT) as a control for 2 days, total RNA was extracted from culture cells, and clusterin and GAPDH levels were analyzed by Northern blotting. No Tx, untreated cells. In B, quantitative analysis of clusterin mRNA levels after normalization to GAPDH mRNA levels in KoTCC-1 cells after treatment with various concentrations of AS clusterin ODN or MM control ODN was performed using laser densitometry. Each point represents the mean of triplicate analyses with SD. * , differs from controls (P < 0.01) by Student’s t test. In C, KoTCC-1 cells were treated daily with 1 μM AS clusterin ODN or MM control ODN for 4 days, protein was extracted from culture cells, and clusterin and β-tubulin protein levels were analyzed by Western blotting. No Tx, untreated cells.

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The abbreviations used are: ODN, oligodeoxynucleotide; AS, antisense; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly(ADP-ribose) polymerase; MM, mismatch; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.
AS Clusterin ODN. The phosphorothioate ODNs used in this study were obtained from Nucleic Acid-Protein Service Unit, University of British Columbia (Vancouver, Canada). The sequences of AS clusterin ODN corresponding to the human clusterin translation initiation site were 5'-H11032-CAGCAGCA-GAGTCTTCATCAT-3'. A 2-base clusterin MM ODN (5'-H11032-CAGCAGCAGAGTATTTA-TCAT-3') was used as a control.

Treatment of Cells with ODN. Lipofectin, a cationic lipid (Life Technologies, Inc.), was used to increase the ODN uptake of cells. KoTCC-1 cells were treated with various concentrations of ODN after a preincubation for 20 min with 3 g/ml lipofectin in serum-free Opti-MEM (Life Technologies, Inc.). After the beginning of the incubation (4 h), the medium containing ODN and lipofectin was replaced with standard culture medium as described above.

Northern Blot Analysis. Total RNA was isolated from cultured KoTCC-1 cells and KoTCC-1 tumor tissues using the acid-guanidium thiocyanate-phenol-chloroform method. Electrophoresis, hybridization, and washing conditions were carried out as reported previously (27). Human clusterin and GAPDH cDNA probes were generated by reverse transcription-PCR from total RNA of human kidney using primers 5'-AA-GGAAATTCAAAATGCTGTCAA-3' (sense) and 5'-ACA-GACAAAGAATCCGCGACTT-3' (AS) for clusterin and 5'-TGCCTTGAACCTCGGTAAGT-3' (sense) and 5'-ATATTT-GGCAGGGTTTCTAGA-3' (AS) for GAPDH. The density of bands for clusterin was normalized against that of GAPDH by densitometric analysis.

Western Blot Analysis. Samples containing equal amounts of protein (15 μg) from lysates of the cultured KoTCC-1 cells and KoTCC-1 tumors were electrophoresed on an 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 1:400-diluted antihuman clusterin goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:10000-diluted antirat β-tubulin mouse monoclonal antibody (Chemicon International, Inc., Tumecula, CA), or 1:600-diluted antihuman PARP mouse monoclonal antibody (PharMingen, Mississauga, Canada). The filters were then incubated for 30 min with horseradish peroxidase-conjugated goat or mouse IgG antibody (Amersham Life Science, Arlington Heights, IL), and specific proteins were detected using an enhanced chemiluminescence Western blotting analysis system (Amersham Life Science).

MTT Assay. The in vitro growth-inhibitory effects of AS clusterin ODN plus cisplatin on KoTCC-1 cells were assessed using the MTT assay as described previously (27). Briefly, 1 × 10⁴ cells were seeded in each well of 96-well microtiter plates and allowed to attach overnight. Cells were then treated once daily with 500 nM ODN for 2 days. After ODN treatment, cells were treated with various concentrations of cisplatin. After 48 h of incubation, 20 μl of 5 mg/ml MTT (Sigma Chemical Co.) in PBS were added to each well, followed by incubation for 4 h at 37°C. The formazan crystals were then 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 for the vehicle-treated cells to determine the percentage of survival. Each assay was performed in triplicate.

DNA Fragmentation Analysis. Nucleosomal DNA degradation was analyzed as described previously with minor modification (27). Briefly, 1 × 10⁵ KoTCC-1 cells were seeded in 5-cm culture dishes and allowed to adhere overnight. After treatment with ODN plus cisplatin using the same schedule described above, cells were harvested and then lysed in a solution containing 100 mM NaCl, 10 mM Tris (pH 7.4), 25 mM EDTA, and 0.5% SDS. After the centrifugation, the superna-
tants were incubated with 300 μg/ml proteinase K for 5 h at 65°C and extracted with phenol-chloroform. The aqueous layer was treated with 0.1 volume of 3 M sodium acetate, and the DNA was precipitated with 2.5 volumes of 95% ethanol. After treatment with 100 μg/ml RNase A for 1 h at 37°C, the sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Animal Studies. Athymic nude mice (BALB/c-nu/nu males, 6–8 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and housed in a controlled environment at 22°C on a 12-h light, 12-h 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. Each of the tumor cell lines was trypsinized, washed twice with PBS, and injected s.c. with 1 × 10^6 cells in the flank or directly administered 1 × 10^6 cells into the bladder wall, as described previously (28).

After injection (10 days), mice were randomly selected for treatment with AS clusterin ODN alone, MM control ODN alone, AS clusterin ODN plus cisplatin, or MM control ODN plus cisplatin. After randomization, 10 mg/kg AS clusterin or MM control ODN were injected i.p. once daily into each mouse for 28 days, and 50 μg of cisplatin were injected i.v. twice a week for 2 weeks. Tumor volume was measured once weekly and calculated by the formula: length × width × depth × 0.5236 (8). After the injection of tumor cells in the bladder wall (8 weeks), the mice were sacrificed, and the presence of metastasis was examined macroscopically in all abdominal and thoracic internal organs. The organs with metastases were removed, and the number of surface metastatic nodules was counted. Data points were reported as average tumor volumes ± SD.

TUNEL Staining. A modified TUNEL technique (8) was used to detect apoptotic cells in KoTCC-1 tumors using the ApopTag In Situ Apoptosis Detection System (Oncor, Gaithersburg, MD) according to the manufacture’s protocol. The percentage of positively stained cells per total number of cells/high power field in five random fields, where no necrotic cells were observed, was counted and averaged.

RESULTS

AS ODN-mediated Inhibition of Clusterin Expression in KoTCC-1 Cells. The effect of treatment with AS clusterin ODN on clusterin mRNA expression in KoTCC-1 cells...
daily treatment of KoTCC-1 cells with AS clusterin ODN (100, 500, or 1000 nM) for 2 days reduced clusterin mRNA levels by 22, 59, or 95%, respectively. In contrast, clusterin mRNA expression was not affected by the 2-base MM control ODN at any of the used concentrations. Inhibition of clusterin protein levels in KoTCC-1 cells was also observed after daily treatment with AS clusterin ODN for 4 consecutive days (Fig. 2C).

**Changes in Clusterin Expression in KoTCC-1 Cells after AS Clusterin ODN and Cisplatin Treatment.** Northern blot analysis was used to determine the effects of cisplatin treatment on clusterin mRNA expression in KoTCC-1 cells. As shown in Fig. 2A, clusterin mRNA induction increased in a dose-dependent manner by cisplatin treatment at concentrations ≤10 mg/ml. Time-course experiments demonstrated that cisplatin-induced clusterin mRNA up-regulation peaked by 48 h post-treatment and began decreasing by 96-h post-treatment (Fig. 2B).

We then examined the effects of combined treatment with AS clusterin ODN and cisplatin on clusterin mRNA expression in KoTCC-1 cells. As shown in Fig. 2C, 500 nM AS clusterin ODN combined with 5 or 10 mg/ml cisplatin decreased clusterin mRNA levels by 81 or 76%, respectively, compared with 500 nM MM control ODN treatment.

**Enhanced Chemosensitivity of KoTCC-1 Cells in Vitro with AS Clusterin ODN Treatment.** To determine whether treatment with AS clusterin ODN enhances the cytotoxic effects of cisplatin, KoTCC-1 cells were treated with 500 nM AS clusterin ODN or MM control ODN once daily for 2 days and then incubated with medium containing various concentrations of cisplatin for 2 days. The MTT assay was then performed to measure the number of viable cells. As shown in Fig. 3A, AS clusterin ODN treatment significantly enhanced chemosensitivity.
Chemosensitization of Bladder Cancer Cells by Antisense Clusterin ODN
during the process of apoptotic execution (29). We addi-
tively evaluated the effects of combined AS clusterin ODN plus cisplatin (Fig. 3C). We addi-
tionally evaluated the effects of combined AS clusterin ODN plus chemotherapy using Western blot analysis to identify
cleavage of PARP protein, a substrate of the caspases activated
during the process of apoptotic execution (29). The Mrc116,000
intact form of PARP was observed in all samples examined,
whereas the Mrc85,000 PARP cleavage fragment was detected
only after combined treatment with AS clusterin ODN plus
cisplatin (Fig. 3D).

Synergistic Inhibition of Growth and Metastasis of
KoTCC-1 Cells in Vivo by AS Clusterin ODN and Cisplatin.
The efficacy of a regimen combining AS clusterin ODN and
and cisplatin for inhibiting the growth of s.c. KoTCC-1 tumors was
evaluated. Athymic nude mice bearing KoTCC-1 tumors ~1 cm
in diameter were randomly selected for treatment with AS
clusterin ODN alone, MM control ODN alone, AS clusterin
ODN plus cisplatin, or MM control ODN plus cisplatin. Mean
tumor volume was similar at the beginning of treatment in each
of these groups. Whereas changes in tumor volume in mice
treated with AS clusterin ODN alone was similar to that with
MM control ODN, KoTCC-1 tumor growth was inhibited
slightly but definitively by treatment with MM control ODN
plus cisplatin, and combined AS clusterin ODN and cisplatin
therapy showed synergistic growth inhibitory effects. After tu-
ror injection (50 days), the tumor volume in mice treated with
AS clusterin ODN plus cisplatin was 69, 68, or 48% smaller
than that in mice treated with AS clusterin ODN alone, MM
control ODN alone, or MM control ODN plus cisplatin, respect-
ively (Fig. 4A). Moreover, TUNEL staining detected a 6-, 6-, or
3-fold increase in the numbers of apoptotic cells in the
KoTCC-1 tumors treated with AS clusterin ODN plus cisplatin
compared with those treated with AS clusterin ODN alone, MM
control ODN alone, or MM control ODN plus cisplatin, respec-
tively (Fig. 4B).

We then performed Northern blot analysis to confirm the
effects of in vivo ODN treatment on clusterin mRNA expression
levels in the KoTCC-1 tumors. In this experiment, each of three
tumor-bearing mice was administered 10 mg/kg AS clusterin or
MM control ODN alone i.p. once daily for 5 days, and on the
next day, tumor tissues were harvested for RNA extraction.
Densitometric analysis revealed that AS clusterin ODN resulted
in a 56% reduction in clusterin mRNA levels in KoTCC-1
tumors compared with MM control ODN-treated tumors (Fig. 4,
C and D).

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KoTCC-1 Cells in Vivo by AS Clusterin ODN and Cisplatin.

### Table 1 Changes in metastasis of KoTCC-1 cells injected into the bladder wall of nude mice after antisense clusterin ODN and/or cisplatin treatment

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Incidence of metastasis (%)</th>
<th>Weight of the primary tumor (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS clusterin ODN</td>
<td>10/10 (100)</td>
<td>10.1</td>
</tr>
<tr>
<td>MM control ODN</td>
<td>10/10 (100)</td>
<td>9.5</td>
</tr>
<tr>
<td>AS clusterin ODN + cisplatin</td>
<td>1/10 (10%)</td>
<td>6/10 (60)</td>
</tr>
<tr>
<td>MM control ODN + cisplatin</td>
<td>6/10 (60)</td>
<td>4/10 (40)</td>
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</tbody>
</table>

- Number of mice with tumor/number of injected mice.
- Two weeks after the implantation of tumor cells, 10 mg/kg AS clusterin ODN or MM clusterin ODN was injected i.p., and 50 μg of cisplatin was injected i.v. twice a week for 2 weeks.
- Number of mice with hemorrhagic ascites/number of 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 < 0.005, Student’s t-test).
progression. Recently, we reported that clusterin mRNA is overexpressed in invasive bladder cancer compared with superficial disease and normal urothelial tissue and that the strong expression of clusterin mRNA in bladder cancer could be used as a prognostic parameter predicting disease recurrence and prognosis (12). However, to our knowledge, there have been no studies analyzing the functional significance of clusterin expression in bladder cancer; therefore, in the present study, we evaluated the effect of decrease in clusterin expression in the human bladder cancer KoTCC-1 cells using AS ODN on apoptosis induced by cisplatin both in vitro and in vivo.

Phosphorothioate AS clusterin ODN corresponding to the human clusterin translation initiation site used in this study inhibited clusterin mRNA expression in a dose- and sequence-dependent manner, even after cisplatin treatment, which resulted in an increase in clusterin expression. Furthermore, treatment of KoTCC-1 cells with AS clusterin ODN reduced the IC\textsubscript{50} of cisplatin by >50% and enhanced cisplatin-induced apoptosis, although no growth inhibitory effects were observed in KoTCC-1 cells treated with AS clusterin ODN alone. These findings suggest that clusterin expression in bladder cancer cells may confer a phenotype resistant to apoptosis induced by chemotherapeutic agents; therefore, despite no significant effect on cell proliferation in the absence of other apoptotic stimuli or cell death signals, the reduction in clusterin expression by AS clusterin ODN may enhance the sensitivity of cytotoxic chemotherapy for bladder cancer.

Most cases of bladder cancer initially respond to cisplatin-based combination chemotherapy; however, the development of an acquired resistant phenotype is observed frequently with the progression of the disease (2). Recently, several investigators have shown that overexpression of antiapoptotic genes in bladder cancer cells, such as mutant-type p53 or Bcl-2, helps mediate drug resistance through the inhibition of apoptosis induced by chemotherapeutic agents (27, 28, 30, 31), suggesting that the approach of enhancing chemosensitivity by decreasing the expression of the antiapoptotic gene appears to be a more rational strategy for patients with advanced bladder cancer than the conventional approach of increasing the dose and combining several kinds of drugs. Furthermore, recent preclinical studies have provided proof of principle evidence that targeting antiapoptotic genes using AS ODN enhances apoptosis induced by conventional cytotoxic chemotherapy (9, 10, 24, 25). Accordingly, based on the findings of the present in vitro experiments, we then examined whether AS clusterin ODN therapy synergistically enhances the cytotoxic effect of cisplatin on the growth and metastasis of KoTCC-1 cells in vivo. Consistent with the in vitro studies, a regimen combining AS clusterin and cisplatin synergistically inhibited the growth of s.c. KoTCC-1 tumors in vivo. These findings suggest that it might be possible to achieve powerful cytotoxic effects of cisplatin at tolerable doses by combining with AS clusterin ODN. The combined regimen also synergistically suppressed the incidence of metastasis after orthotopic injection of KoTCC-1 cells, resulting in a significant delay of tumor progression. Although it remains unclear whether this combined regimen directly affects the metastatic process or suppresses metastasis through inhibition of the growth of the orthotopic tumors, it might be efficacious to use the combined AS clusterin ODN and cisplatin therapy as a prophylactic adjuvant therapy against postoperative metastasis.

In conclusion, these findings in the present study suggest that expression of clusterin helps mediate bladder cancer progression by inhibiting apoptotic cell death induced by several kinds of therapy, including cytotoxic chemotherapy. Decreasing clusterin-mediated chemoresistance by AS clusterin ODN may provide a feasible and safe strategy to enhance chemosensitivity in bladder cancer. The preclinical data presented here provides proof of principle support for designing clinical studies with combined AS clusterin ODN plus cisplatin therapy for patients with advanced disease.

REFERENCES


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