Synergistic Chemsensitization and Inhibition of Tumor Growth and Metastasis by the Antisense Oligodeoxynucleotide Targeting Clusterin Gene in a Human Bladder Cancer Model[1]

Hideaki Miyake,[2] Isao Hara, Sadao Kamidono, and Martin E. Gleave

The Prostate Centre, Vancouver General Hospital, Vancouver, British Columbia, V6H 3Z6 Canada [H. M., M. E. G.]; Division of Urology, University of British Columbia, Vancouver, British Columbia, V5Z 3J5 Canada [H. M., M. E. G.]; and Department of Urology, Kobe University School of Medicine, Chuo-ku, Kobe 650-0017, Japan [H. M., I. H., S. K.]

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 pathophysiological 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.

Received 7/3/01; revised 9/6/01; accepted 9/6/01.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by Grant 009002 from the National Cancer Institute of Canada and by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.
[2] To whom requests for reprints should be addressed, at the Department of Urology, Kobe University School of Medicine, Chuo-ku, Kobe 650-0017, Japan.

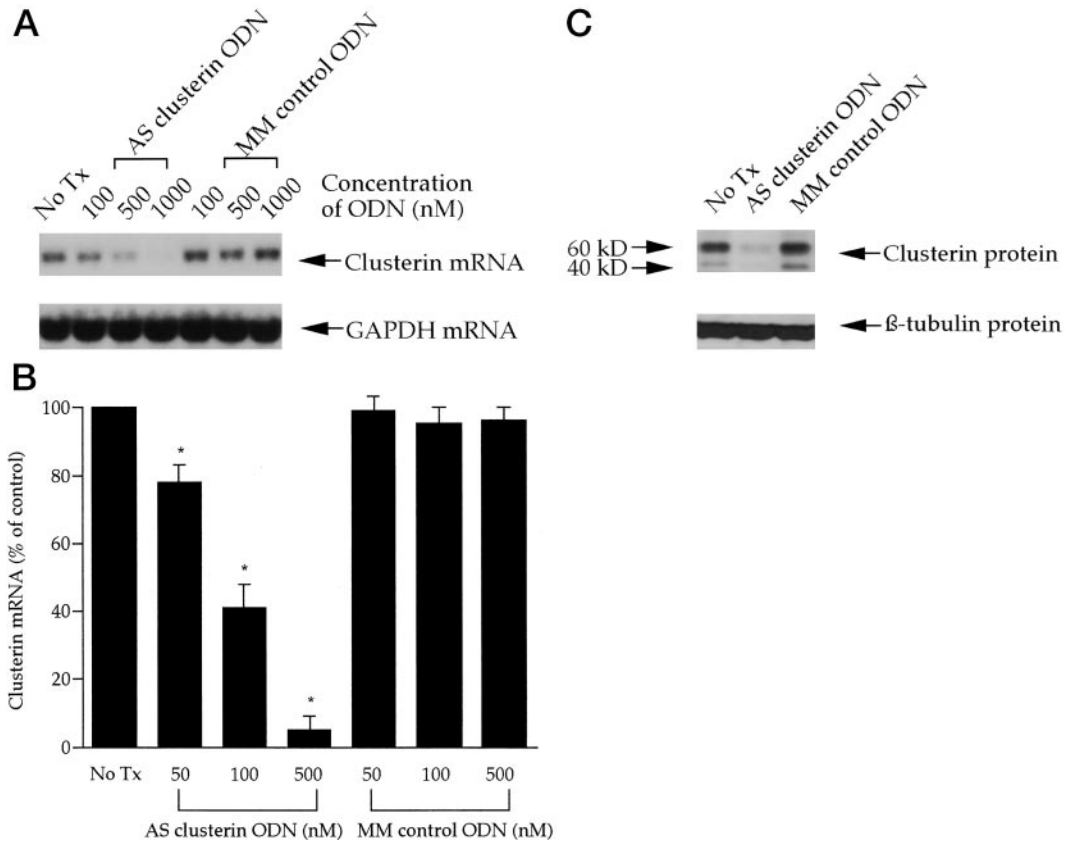

Fig. 1 Sequence-specific and dose-dependent inhibition of clusterin expression by AS clusterin ODN in KoTCC-1 cells. In *A*, KoTCC-1 cells were treated daily with various concentrations of AS cluserin ODN (CAGCAGCAGAGTCTTCATCAT) or a 2-base clusterin MM ODN (CAGCAGCAGAGTATTTATCAT) as a control for 2 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.

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[3]-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.

[3] 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.

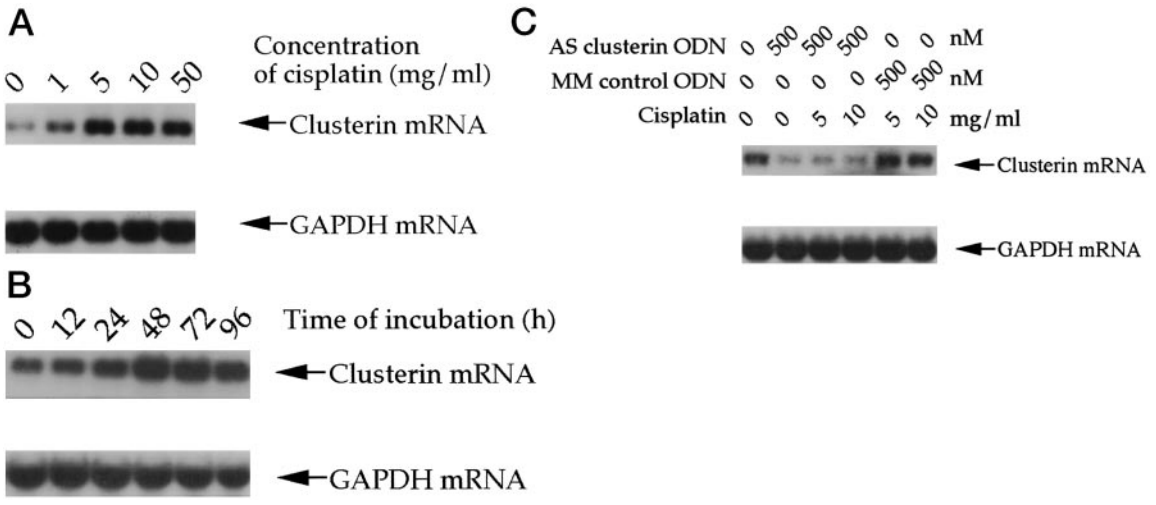

Fig. 2 Effects of AS clusterin ODN and/or cisplatin treatment on clusterin expression in KoTCC-1 cells. In *A*, cells were treated with various concentrations of clisplatin for 48 h; total RNA was then extracted and analyzed for clusterin and GAPDH levels by Northern blotting. In *B*, cells were treated with 10 mg/ml cisplatin for indicated intervals, total RNA was then extracted, and clusterin and GAPDH levels were analyzed by Northern blotting. In *C*, cells were treated daily with 500 nM AS clusterin ODN or a 2-base clusterin MM control ODN for 2 days. After a 24-h exposure to 5 or 10 mg/ml cisplatin, total RNA was then extracted, and clusterin and GAPDH levels were analyzed by Northern blotting.

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′-CAGCAGCA-GAGTCTTCATCAT-3′. A 2-base clusterin MM ODN (5′-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 μg/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-GACAAGATCTCCCGGCACTT-3′ (AS) for clusterin and 5′-TGCTTTTAACTCTGGTAAAGT-3′ (sense) and 5′-ATATTT-GGCAGGTTTTTCTAGA-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 a 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 anti-human PARP mouse monoclonal antibody (PharMingen, Mississauga, Canada). The filters were then incubated for 30 min with horseradish peroxidase-conjugated antigoat 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, 1×10^4 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, 1×10^5 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-

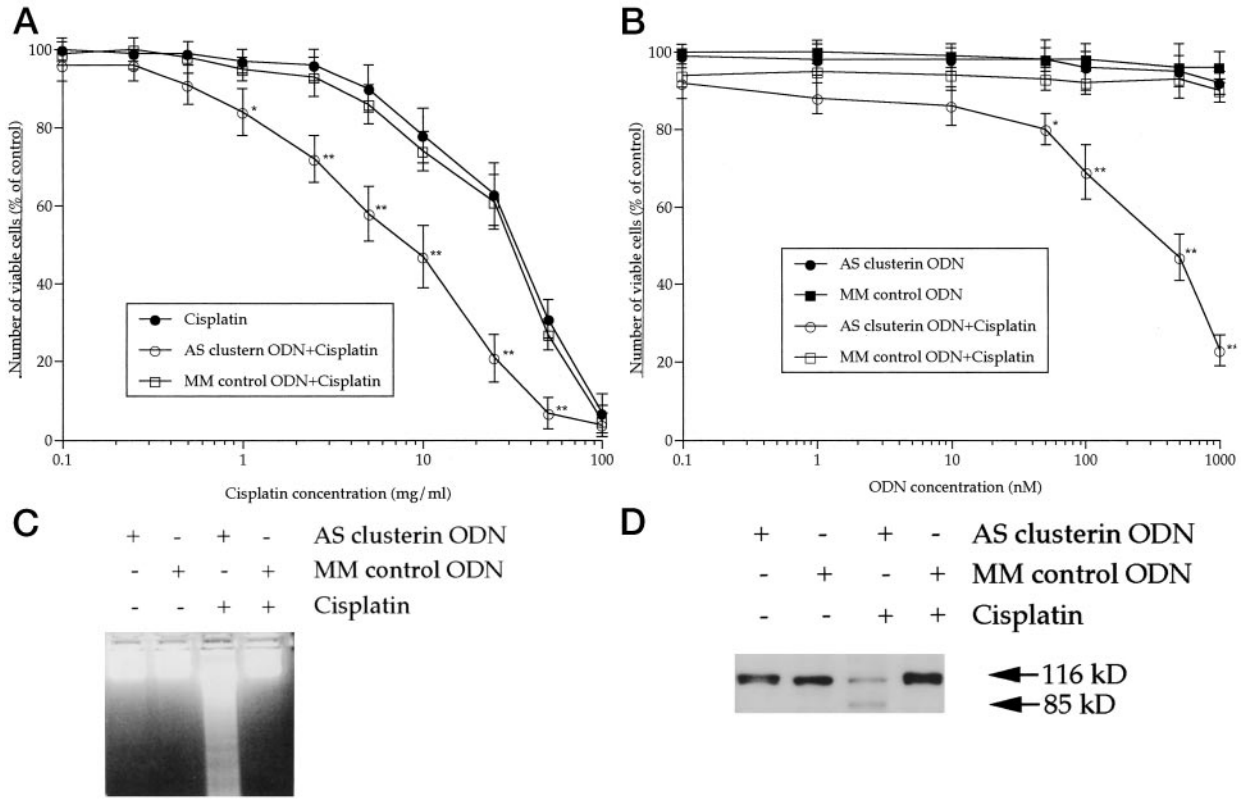

Fig. 3 Effect of combined treatment with AS clusterin ODN and cisplatin on KoTCC-1 cell growth and apoptosis. In *A*, KoTCC-1 cells were treated daily with 500 nM AS clusterin ODN or MM control ODN for 2 days. After ODN treatment, the medium was replaced with medium containing various concentrations of cisplatin. After 48 h of incubation, the number of viable cells was determined by the MTT assay. Each data point represents the mean of triplicate analyses with SD. ** and *, differs from controls ($P < 0.01$ and $P < 0.05$, respectively) by Student's *t* test. In *C*, after the same treatment schedule as described in *A* and *B*, DNA was extracted from KoTCC-1 cells, electrophoresed in a 2% agarose gel, and visualized by ethidium bromide staining and UV transillumination. In *D*, proteins were extracted from KoTCC-1 cells after the same treatment as described in *A* and *B* and analyzed by Western blotting with an anti-PARP antibody. Uncleaved intact PARP, M_r = 116,000; cleaved PARP, M_r = 85,000.

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* 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-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

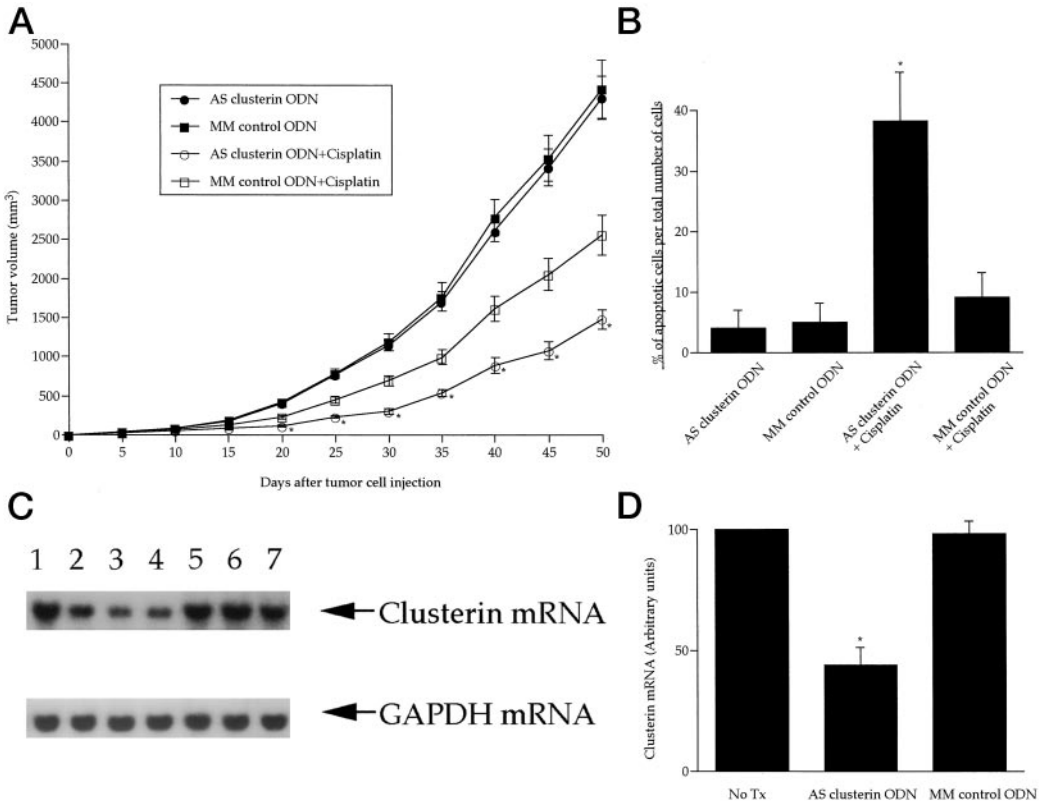

Fig. 4 Effects of combined treatment with AS clusterin ODN plus cisplatin on KoTCC-1 tumor growth. In *A*, mice bearing KoTCC-1 tumor were randomly selected for treatment with AS clusterin ODN, MM control ODN, AS clusterin ODN plus cisplatin, or MM control ODN plus cisplatin. After tumor cell injection (10 days), 10 mg/kg AS clusterin ODN or MM control ODN was daily injected i.p. for 28 days. Cisplatin (50 μg) was 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. Each point represents the mean tumor volume in each experimental group containing eight mice with SD. ∗, differs from controls ($P < 0.01$) by Student's *t* test. In *B*, 30 days after tumor cell injection, KoTCC-1 tumors were harvested from each treatment group for the detection of apoptosis using TUNEL staining. The percentage of positively stained cells per total number of cells/high power field in five random fields was counted and averaged. ∗, differs from the other treatment groups ($P < 0.01$) by Student's *t* test. In *C* and *D*, each of three KoTCC-1 tumor-bearing mice were treated daily with AS clusterin ODN or MM control ODN at a dose of 10 mg/kg for 5 days, total RNA was extracted from KoTCC-1 tumors 6 days after the initiation of treatment, and clusterin and GAPDH mRNA levels were analyzed by Northern blotting. *Lane 1*, control KoTCC-1 tumor without any treatment; *Lanes 2–4*, KoTCC-1 tumors in mice administered AS clusterin ODN; *Lanes 5–7*, KoTCC-1 tumors in mice administered MM control ODN (*C*). Quantitative analysis of clusterin mRNA levels after normalization to GAPDH mRNA levels in KoTCC-1 tumors after treatment with AS clusterin ODN or MM control ODN was performed using a laser densitometer. Each *column*, the mean value with SD. ∗, differs from controls ($P < 0.01$) by Student's *t* test (*D*).

was evaluated by Northern blot analysis. As shown in Fig. 1, *A* and *B*, 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 clustern protein levels in KoTCC-1 cells was also observed after daily treatment with AS clusterin ODN for 4 consecutive days (Fig. 2*C*).

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. 2*A*, 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. 2*B*).

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. 2*C*, 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. 3*A*, AS clusterin ODN treatment significantly enhanced chemosensitiv-

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

Regimen[b]	Incidence of metastasis (%)[a]			Weight of the primary tumor (mg)
	Retroperitoneal lymph node metastasis	Intraabdominal lymph node metastasis	Incidence of hemorrhagic ascites[c]	
AS clusterin ODN	10/10 (100)	7/10 (70)	6/10 (60)	39.2 ± 9.5[d]
MM control ODN	10/10 (100)	9/10 (90)	7/10 (70)	41.1 ± 10.1
AS clusterin ODN + cisplatin	1/10 (10)[e]	0/10 (0)[e]	0/10 (0)[e]	20.6 ± 7.1[f]
MM control ODN + cisplatin	6/10 (60)	5/10 (50)	4/10 (40)	33.6 ± 9.2

[a] Number of mice with tumor/number of injected mice.
[b] 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.
[c] Number of mice with hemorrhagic ascites/number of injected mice.
[d] Mean ±SD.
[e] The incidence of metastasis or hemorrhagic ascites was significantly different from that in mice treated with other regimens ($P < 0.05$, χ^d test).
[f] 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).

ity of cisplatin in a dose-dependent manner, reducing the IC_{50} of cisplatin by >50%. Dose-dependent synergy between AS clusterin ODN and cisplatin was also observed by increasing the AS ODN concentration when the cisplatin concentration was fixed at 10 mg/ml (Fig. 3*B*).

The DNA fragmentation assay was performed to compare induction of apoptosis after treatment with 500 nM AS clusterin ODN either alone or in combination with 5 nM cisplatin. Using the same treatment schedule described above, the characteristic apoptotic DNA ladder was observed only after combined treatment of AS clusterin ODN plus cisplatin (Fig. 3*C*). We additionally 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 M_r 116,000 intact form of PARP was observed in all samples examined, whereas the M_r 85,000 PARP cleavage fragment was detected only after combined treatment with AS clusterin ODN plus cisplatin (Fig. 3*D*).

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 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 tumor 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, respectively (Fig. 4*A*). 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, respectively (Fig. 4*B*).

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*).

We finally examined the effects of the combined regimen using a recently reported orthotopic injection (*i.e.*, bladder wall injection) model (28), according the same treatment schedule described above. As shown in Table 1, the combined AS clusterin and cisplatin therapy substantially suppressed the primary tumor growth as well as the incidence of metastasis after orthotopic injection of KoTCC-1 cells compared with the other three treatment regimens. Both the weight of primary KoTCC-1 tumor and the incidences of retroperitoneal lymph node metastasis, mesenteric lymph node metastasis, and hemorrhagic ascites in mice treated with combined AS clusterin ODN and cisplatin were significantly lower than those in mice treated with AS clusterin ODN alone, MM control ODN alone, or MM control ODN plus cisplatin.

DISCUSSION

Despite the original hypothesis that clusterin is a marker for programmed cell death (3–5), several experimental and clinical studies have demonstrated conflicting findings indicating the dissociation of clusterin expression from apoptosis (6–11). We also demonstrated previously that overexpression of clusterin helps mediate androgen-independent progression against castration- and chemotherapy-induced apoptosis using several prostate cancer models (8–11). Collectively, these findings suggest that clusterin up-regulation plays a protective role against apoptosis induced by various kinds of stimuli and thereby may confer an aggressive phenotype during cancer

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_{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

1. Thurman, S. A., and DeWeese, T. L. Multimodality therapy for the treatment of muscle-invasive bladder cancer. Semin. Urol. Oncol., *18:* 313–322, 2000.

2. Vaughn, D. J., and Malkowicz, S. B. Recent advances in bladder cancer chemotherapy. Cancer Investig., *19:* 77–85, 2001.

3. Rosenberg, M. E., and Silkensen, J. Clusterin: physiologic and pathophysiologic considerations. Int. J. Biochem. Cell Biol., *27:* 633–645, 1995.

4. Connor, J., Buttyan, R., Olsson, C. A., D'Agati, V., O'Toole, K., and Sawczuk, I. S. SGP-2 expression as a genetic marker of progressive cellular pathology in experimental hydronephrosis. Kidney Int., *39:* 1098–1103, 1991.

5. Kyprianou, N., English, H. F., Davidson, N. E., and Isaaca, J. T. Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. Cancer Res., *51:* 162–166, 1991.

6. French, L. E., Sappio, A. P., Tschopp, J., and Schifferli, J. A. Distinct sites of production and deposition of the putative cell death marker clusterin in the human thymus. J. Clin. Investig., *90:* 1919–1925, 1992.

7. Sensibar, J. A., Sutkowski, D. M., Raffo, A., Buttyan, R., Griswold, M. D., Sylvester, S. R., Kozlowski, J. M., and Lee, C. Prevention of cell death induced by tumor necrosis factor α in LNCaP cells by overexpression of sulfated glycoprotein-2 (clusterin). Cancer Res., *55:* 2431–2437, 1995.

8. Miyake, H., Nelson, C., Rennie, P. S., and Gleave, M. E. Testosterone-repressed prostate message-2 is an antiapoptotic gene involved in progression to androgen-independence in prostate cancer. Cancer Res., *60:* 170–176, 2000.

9. Miyake, H., Nelson, C., Rennie, P. S., and Gleave, M. E. Acquisition of chemoresistant phenotype by overexpression of the antiapoptotic gene, testosterone-repressed prostate message-2, in prostate cancer xenograft models. Cancer Res., *60:* 2547–2554, 2000.

10. Miyake, H., Chi, K. N., and Gleave, M. E. Antisense TRPM-2 oligodeoxynucleotides chemosensitize human androgen-independent PC-3 prostate cancer cells both *in vitro* and *in vivo*. Clin. Cancer Res., *6:* 1655–1663, 2000.

11. Miyake, H., Hara, I., Kamidono, S., and Gleave, M. E. Novel therapeutic strategy for advanced prostate cancer using antisense oligodeoxynucleotides targeting antiapoptotic genes up-regulated after androgen withdrawal to delay androgen-independent progression and enhance chemosensitivity. Int. J. Urol., *8:* 337–349, 2001.

12. Miyake, H., Gleave, M., Kamidono, S., and Hara, I. Overexpression of clusterin in transitional cell carcinoma of the bladder is related to disease progression and recurrence. Urology, in press.

13. Duggan, B. J., Kelly, J. D., Keane, P. F., and Johnston, S. R. Molecular targets for the therapeutic manipulation of apoptosis in bladder cancer. J. Urol., *165:* 946–954, 2001.

14. Inoue, K., Perrotte, P., Wood, C. G., Slaton, J. W., Sweeney, P., and Dinney, C. P. Gene therapy of human bladder cancer with adenovirus-mediated antisense basic fibroblast growth factor. Clin. Cancer Res., *6:* 4422–4431, 2000.

15. Miyake, H., Yoshimura, K., Hara, I., Eto, H., Arakawa, S., and Kamidono, S. Basic fibroblast growth factor regulates matrix metalloproteinases production and *in vitro* invasiveness in human bladder cancer cell lines. J. Urol., *157:* 2351–2355, 1997.

16. Koga, S., Kondo, Y., Komata, T., and Kondo, S. Treatment of bladder cancer cells *in vitro* and *in vivo* with 2–5A antisense telomerase RNA. Gene Ther., *8:* 654–658, 2001.

17. Miyake, H., Hara, I., Yamanaka, K., Arakawa, S., and Kamidono, S. Calcium ionophore, ionomycin inhibits growth of human bladder cancer cells both *in vitro* and *in vivo* with alteration of Bcl-2 and Bax expression levels. J. Urol., *162:* 916–921, 1999.

18. Miyake, H., Hara, I., Hara, S., Arakawa, S., and Kamidono, S. Synergistic chemosensitization and inhibition of tumor growth and metastasis by adenovirus-mediated p53 gene transfer in human bladder cancer model. Urology, *56:* 332–336, 2000.

19. Wagner, R. W. The state of the art in antisense research. Nat. Med., *1:* 1116–1118, 1995.

20. Monia, B. P., Johnston, J. F., Geiger, T., Muller, M., and Fabbro, D. Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against C-raf kinase. Nat. Med., *2:* 668–675, 1996.

21. Cucco, C., and Calabretta, B. *In vitro* and *in vivo* reversal of multidrug resistance in a human leukemia-resistant cell line by mdr1 antisense oligodeoxynucleotides. Cancer Res., *56:* 4332–4337, 1996.

22. Miyake, H., Tolcher, A., and Gleave, M. E. Antisense Bcl-2 oligodeoxynucleotides inhibit progression to androgen-independence after castration in the Shionogi tumor model. Cancer Res., *59:* 4030–4034, 1999.

23. Miyake, H., Pollak, M., and Gleave, M. E. Castration-induced up-regulation of insulin-like growth factor binding protein-5 potentiates insulin-like growth factor-I activity and accelerates progression to androgen independence in prostate cancer models. Cancer Res. *60:* 3058–3064, 2000.

24. Geiger, T., Muller, M., Monia, B. P., and Fabbro, D. Antitumor activity of a C-raf antisense oligodeoxynucleotide in combination with standard chemotherapeutic agents against various human tumors transplanted subcutaneously into nude mice. Clin. Cancer Res., *3:* 1179–1185, 1997.

25. Jansen, B., Schlagbauer-Wadl, H., Brown, B. D., Bryan, R. N., van Elisas, A., Muller, M., Wolff, K., Eichler, H. G., and Pehamberger, H. bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. Nat. Med., *4:* 232–234, 1998.

26. Miyake, H., Tolcher, A., and Gleave, M. E. Chemosensitization and delayed androgen-independent recurrence of prostate cancer with the use of antisense Bcl-2 oligodeoxynucleotides. J. Natl. Cancer Inst. (Bethesda), *92:* 34–41, 2000.

27. Miyake, H., Hanada, N., Nakamura, H., Kagawa, S., Fujiwara, T., Hara, I., Eto, H., Gohji, K., Arakawa, S., Kamidono, S., and Saya, H. Overexpression of Bcl-2 in bladder cancer cells inhibits apoptosis induced by cisplatin and adnoviral-mediated p53 gene transfer. Oncogene, *16:* 933–943, 1998.

28. Miyake, H., Hara, I., Yamanaka, K., Gohji, K., Arakawa, S., and Kamidono, S. Overexpression of Bcl-2 enhances metastatic potential of human bladder cancer cells. Br. J. Cancer, *79:* 1651–1656, 1999.

29. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature, *371:* 346–347, 1994.

30. Kakehi, Y., Ozdemir, E., Habuchi, T., Yamabe, H., Hashimura, T., Katsura, Y., and Yoshida, A. Absence of p53 overexpression and favorable response to cisplatin-based neoadjuvant chemotherapy in urothelial carcinomas. Jpn. J. Cancer Res., *89:* 214–220, 1998.

31. Miyake, H., Hara, I., Yamanaka, K., Arakawa, S., and Kamidono, S. Synergistic enhancement of resistance to cisplatin in human bladder cancer cells by overexpression of mutant-type p53 and Bcl-2. J. Urol., *162:* 2176–2181, 1999.

Clinical Cancer Research

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

Hideaki Miyake, Isao Hara, Sadao Kamidono, et al.

Clin Cancer Res 2001;7:4245-4252.