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Introduction of *Clusterin* Gene into Human Renal Cell Carcinoma Cells Enhances Their Resistance to Cytotoxic Chemotherapy through Inhibition of Apoptosis both *in vitro* and *in vivo*

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Recent studies have revealed the powerful antiapoptotic activity of clusterin in various malignant tumors; however, the significance of clusterin expression in the acquisition of a resistant phenotype against several kinds of treatment in human renal cell carcinoma (RCC) has not been well characterized. We, therefore, transfected the *clusterin* cDNA into RCC ACHN cells, that scarcely express clusterin protein, to examine whether overexpression of clusterin inhibits chemotherapy-induced apoptosis both *in vitro* and *in vivo*. Although no significant differences were observed in the *in vitro* growth rates between clusterin-transfected ACHN (ACHN/CL) and the vector only-transfected cell line (ACHN/Co), ACHN/CL exhibited high resistance to cisplatin treatment compared with ACHN/Co, with a greater than 5-fold higher IC_{50} through the inhibition of apoptotic cell death, which was demonstrated by DNA fragmentation analysis and western blotting of PARP protein. Moreover, intravenous administration of cisplatin into athymic nude mice bearing ACHN/CL tumors resulted in 2- to 3-times faster tumor growth compared with ACHN/Co tumors. These findings suggest that clusterin overexpression helps confer a chemoresistant phenotype through inhibition of apoptosis in human RCC cells.

Key words: Clusterin — Chemotherapy — Renal cell carcinoma — Apoptosis

Renal cell carcinoma (RCC) is the most common malignancy of the adult kidney, and the 10th leading cause of cancer-related death in Western industrialized countries. Due to the highly resistant phenotype to conventional therapies, including radiation and chemotherapy, surgical resection of localized disease has been the only curative treatment; therefore, the prognosis of patients with invasive and/or metastatic diseases remains generally poor.[1)] Moreover, different from other malignant tumors, there was no significant association between the prognosis of patients with RCC and the expression of apoptosis-related molecules, such as p53, Bcl-2, Bax and c-Myc.[2–5)] Accordingly, the mechanism of resistance to therapy-induced apoptosis in RCC remains largely unknown.

Clusterin, also known as testosterone-repressed prostate message-2 or sulfated glycoprotein-2, was first isolated from ram rete testes fluid, and plays crucial roles in various pathophysiological processes, including tissue remodeling, reproduction, lipid transport, complement regulation and apoptosis.[6)] Since clusterin expression is increased in various benign and malignant tissues undergoing apoptosis, it has been regarded as a marker for apoptotic cell death.[7,8)] Recent studies, however, have provided conflicting findings regarding the relationship between up-regulated clusterin expression and increased apoptotic activity.[9,10)] Recently, we also demonstrated the powerful antiapoptotic activity of clusterin using several kinds of prostate cancer models; that is, increase in clusterin expression after androgen withdrawal stimulates tumor progression by inhibiting castration- and chemotherapy-induced apoptosis.[11–14)] Despite the finding by Parczyk *et al.* that clusterin mRNA is 3-fold overexpressed in tumor tissues compared with adjacent normal tissue,[15)] the functional significance of clusterin expression in RCC has not been well characterized.

In the present study, we, therefore, introduced *clusterin* cDNA into ACHN cells, a human RCC cell line that scarcely expresses the clusterin protein, to examine whether clusterin overexpression confers resistance to apoptotic cell death induced by cytotoxic chemotherapy.

MATERIALS AND METHODS

Tumor cell line ACHN derived from human RCC was purchased from the American Type Culture Collection (Rockville, MD). It was maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 0.1 m*M* non-essential amino acids and 1 m*M* sodium pyruvate.

Expression plasmid and transfection to tumor cells pRC-CMV expression vector containing the 1.6 kb cDNA

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fragment encoding human clusterin was provided by Dr. Martin Tenniswood (W. Alton Jones Cell Science Center, Lake Placid, NY). The expression vector was transfected into ACHN cells by the liposome-mediated gene transfer method.[11] Briefly, 2×10^5 ACHN cells were plated in 6-cm plates. The following day, 5 μg of purified clusterin cloned pRC-CMV or pRC-CMV alone (as a control) was added to ACHN cells after preincubation for 30 min with 5 μg of lipofectamine reagent and 3 ml of serum-free OPTI-MEM (Life Technologies, Inc., Gaithersburg, MD). Drug selection, in 300 μg/ml Geneticin (Sigma Chemical Co., St. Louis, MO), was begun 3 days after the transfection. Colonies were harvested 2 weeks after drug selection using cloning cylinders and expanded to cell lines.

Northern blot analysis Total RNA was isolated from the ACHN sublines by the acid-guanidium thiocyanate-phenol-chloroform method, and 20 μg of total RNA from each cell line was subjected to electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nylon membranes (Amersham Life Science, Arlington Heights, IL) overnight according to the standard procedure.[16] The RNA blots were hybridized with a human *clusterin* cDNA probe labeled with [^{32}P]dCTP by random primer labeling. After stripping, the membranes were rehybridized with a mouse glyceraldehyde 3′-phosphate dehydrogenase (GAPDH) cDNA probe. These probes were generated by reverse transcription-PCR from total RNA of human kidney using the previously reported primers.[13] The density of clusterin bands 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 ACHN cells were electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to a nitrocellulose filter. The filters were blocked overnight in phosphate-buffered saline (PBS) containing 5% nonfat milk powder at 4°C and then incubated for 1 h with a 1:600-diluted anti-human PARP mouse monoclonal antibody (Pharmingen, Mississauga, Canada). The filters were then incubated for 30 min with horseradish peroxidase-conjugated mouse IgG antibody (Amersham Life Science), and specific proteins were detected using an enhanced chemiluminescence western blotting analysis system (Amersham Life Science).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay The *in vitro* growth-inhibitory effects of cisplatin on the ACHN sublines were assessed using the MTT assay as described previously.[13] Briefly, 1×10^4 cells were seeded in each well of 96-well microtiter plates and allowed to attach overnight. Cells were then treated with various concentrations of cisplatin, a generous gift from Nippon Kayaku Co. (Tokyo). After 48 h of incubation, 20 μl of 5 mg/ml MTT (Sigma Chemical Co.) in PBS was added to each well, followed by incubation for 4 h at 37°C. The formazan crystals were dissolved in dimethylsulfoxide (DMSO) and the optical density 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 percent survival. Each assay was performed in triplicate.

DNA fragmentation analysis Nucleosomal DNA degradation was analyzed as described previously with a minor modification.[14] Briefly, 1×10^5 cells of the ACHN sublines were seeded in 5-cm culture dishes and allowed to adhere overnight. After treatment with cisplatin using the same schedule described above, cells were harvested and then lysed in a solution containing 100 m*M* NaCl, 10 m*M* Tris pH 7.4, 25 m*M* EDTA, and 0.5% SDS. After the centrifugation, the supernatants 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. Following 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.

Assessment of *in vivo* tumor growth Approximately 1×10^6 cells of each ACHN subline were inoculated subcutaneously with 0.1 ml of Matrigel (Becton Dickinson Labware, Bedford, MA) in the flank region of 6- to 8-week-old male athymic nude mice (BALB/c strain; CLEA Japan, Inc., Tokyo) under methoxyfluorane anesthesia. Four weeks after tumor cell injection, 50 μg/mouse of cisplatin was injected intravenously twice a week for 2 weeks. Each experimental group consisted of 8 mice. Tumor volume was measured once weekly and calculated by using the formula length×width×depth×0.5236.[13] Data points were reported as average tumor volumes±SD.

RESULTS

Generation of clusterin-overexpressing ACHN cell lines ACHN cells were transfected with the human clusterin cDNA expression vector pRc-CMV/Clusterin or the pRc-CMV vector alone as a control. After the drug selection, several Geneticin-resistant stable transfectants were isolated, and analyzed for expression of the clusterin RNA by northern blotting. As shown in Fig. 1, clusterin RNA was detected in 4 independent clusterin-transfected cell lines (ACHN/CL#1 to ACHN/CL#4). No detectable clusterin RNA was expressed in either the parental ACHN (ACHN/P) or the control vector-transfected cell line (ACHN/Co). The four clones expressing high levels of clusterin RNA showed similar results in subsequent *in vitro* experiments; therefore, we hereafter report only the data for ACHN/P, ACHN/Co, ACHN/CL#1, and ACHN/CL#2.

***In vitro* effects of cisplatin treatment on ACHN sublines** To determine whether clusterin overexpression con-

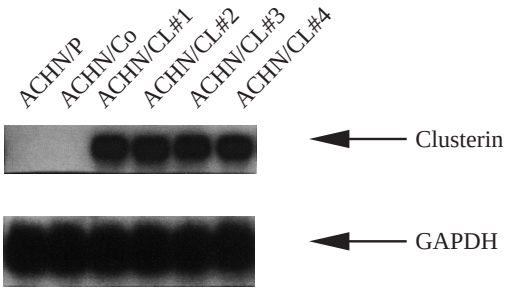

Fig. 1. Northern blot analysis of clusterin mRNA expression in the ACHN sublines. Total RNA was extracted from ACHN/P (parental cell line of ACHN), ACHN/Co (vector only-transfected cell line), and 4 clones of clusterin transfectants (LNCaP/CL#1 to LNCaP/CL#4), and analyzed for clusterin and GAPDH mRNA expression levels by hybridization with a radiolabeled clusterin and GAPDH cDNA fragments, respectively.

fers a proliferative advantage on ACHN, the *in vitro* growth rates of ACHN/P, ACHN/Co, ACHN/CL#1 and ACHN/CL#2 were analyzed using the MTT assay. There was no significant difference in cell proliferation among these cell lines (data not shown).

The ACHN sublines were subsequently examined for their response to cisplatin *in vitro*. ACHN/CL#1 and ACHN/CL#2 acquired resistance to cisplatin in comparison with ACHN/P and ACHN/Co (Fig. 2A); that is, overexpression of clusterin in ACHN cells increased the IC_{50} of cisplatin more than 5-fold.

The induction of apoptosis in ACHN sublines treated with 10 μg/ml cisplatin for 72 h was assessed by DNA degradation assay and western blot analysis of PARP protein, a substrate of the caspases activated during the process of apoptotic execution.[17] The characteristic apoptotic DNA ladders were detected in ACHN/P and ACHN/Co, but not in ACHN/CL#1 or ACHN/CL#2 (Fig. 2B). Similarly, the *Mr* 116 000 intact form of PARP was observed in all ACHN sublines, whereas the *Mr* 85 000 PARP cleavage fragment was detected after cisplatin treatment only in ACHN/P and ACHN/Co (Fig. 2C).

Acquisition of a resistant phenotype to cisplatin by overexpression of clusterin in ACHN tumors *in vivo* To examine the *in vivo* effect of clusterin expression on tumor

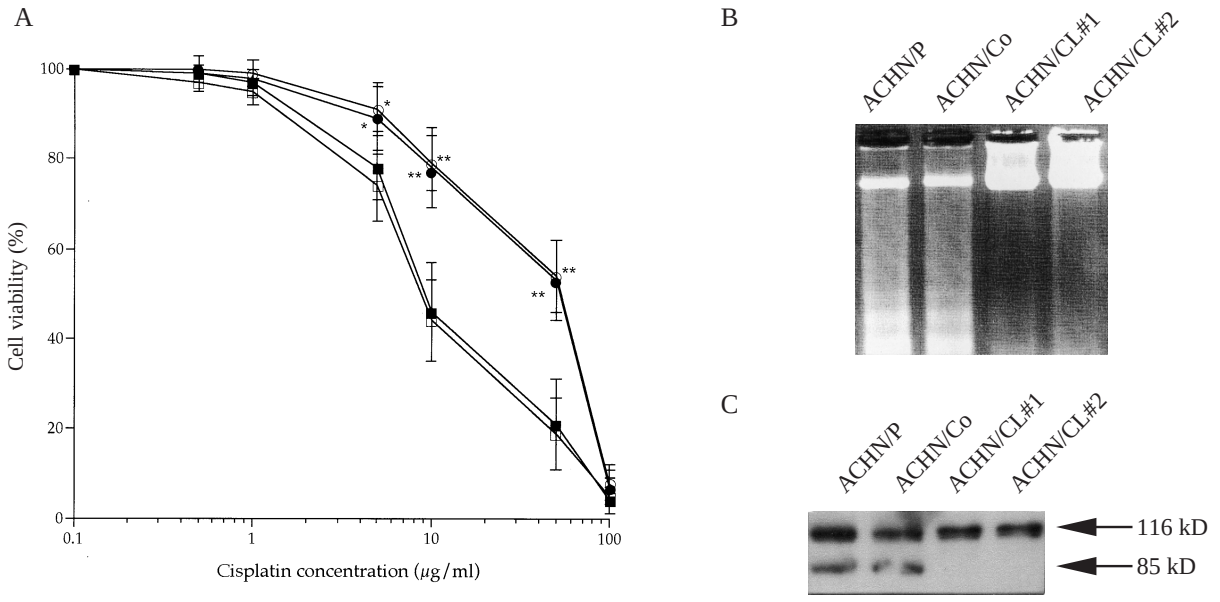

Fig. 2. (A) Effect of cisplatin on the proliferation of the ACHN parental cell line (ACHN/P), the control vector-transfected cell line (ACHN/Co), and clones of clusterin-transfected ACHN (ACHN/CL#1 and ACHN/CL#2). One thousand cells of each cell line were seeded in 96-well microtiter plates and allowed to attach overnight. Then tumor cells were treated with various concentrations of cisplatin for 48 h, and cell viability was determined by the MTT assay. Each point represents the mean of triplicate analyses with SD. * and **, differ from ACHN/P and ACHN/Co ($P<0.05$ and $P<0.01$, respectively) by Student's *t* test. ■, ACHN/P; □, ACHN/Co; ●, ACHN/CL#1; ○, ACHN/CL#2. (B) DNA fragmentation assay of the ACHN sublines treated with cisplatin. The ACHN sublines were treated with 10 μg/ml cisplatin. After 48 h of incubation, DNA was extracted from each cell line, electrophoresed in a 2% agarose gel, and visualized by ethidium bromide staining and UV transillumination. (C) Proteins were extracted from ACHN cells after the same treatment as described in (B), and analyzed by western blotting with an anti-PARP antibody. Uncleaved intact PARP, *Mr*=116 000; cleaved PARP, *Mr*=85 000.

growth, 5×10^6 cells of each cell line were injected subcutaneously in the flank of athymic nude mice. There were no significant differences in tumor growth among ACHN/P, ACHN/Co, ACHN/CL#1 and ACHN/CL#2 (data not shown); that is, the clusterin overexpression did not stimulate the proliferative potential of ACHN *in vivo*, as well as *in vitro*.

To evaluate the sensitivities of the ACHN sublines to cisplatin treatment *in vivo*, 4 weeks after tumor cell injection, 50 μg/mouse of cisplatin was injected intravenously twice a week for 2 weeks. Sixty days after the injection of tumor cells, ACHN/CL#1 and ACHN/CL#2 formed tumors more than twice as large as those formed by ACHN/P and ACHN/Co (Fig. 3). Under the experimental conditions used in the above experiment, no tumor-related deaths were observed.

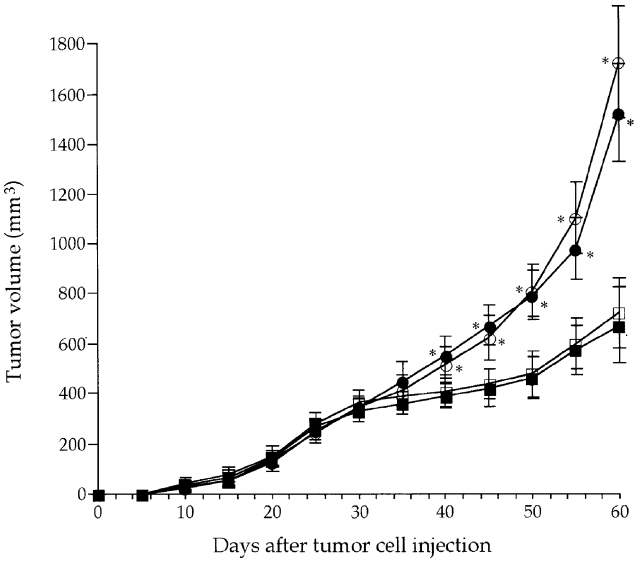

Fig. 3. Effect of cisplatin treatment on tumor growth of the ACHN sublines in nude mice. Four weeks after tumor cell injection, 50 μg/mouse of cisplatin was administered intravenously twice a week for 2 weeks. Tumor volume was measured twice a week and calculated using the formula length×width×depth×0.5236. Each point represents the mean tumor volume in each experimental group containing 8 mice with SD. *, differs from ACHN/P and ACHN/Co ($P<0.01$) by Student's *t* test. ■, ACHN/P; □, ACHN/Co; ●, ACHN/CL#1; ○, ACHN/CL#2.

DISCUSSION

RCC is known for its unusual clinical features. For example, metastases of RCC can spontaneously regress after removal of the primary tumor, and metastasis can occur many years after nephrectomy. In addition, RCC is characterized as highly resistant to several kinds of therapeutic options, including chemotherapy and radiation.[1] However, the precise mechanism of the therapy-resistant phenotype in RCC has not been well demonstrated. In fact, recent studies reported that apoptosis-related molecules, such as p53, Bcl-2, Bax and c-Myc, play no functional role in the progression of RCC.[2–5] These findings suggest that RCC may acquire antiapoptotic activity against several therapies through a unique mechanism.

Clusterin was initially considered as a marker and putative mediator of apoptotic cell death, because its expression is substantially up-regulated in various normal and malignant tissues undergoing apoptosis.[7, 8] In recent studies, however, clusterin up-regulation has been shown to be dissociated from apoptosis.[9, 10] We also demonstrated the antiapoptotic activity of clusterin against androgen ablation and cytotoxic chemotherapy using several kinds of prostate cancer models.[11–14] In the kidney, up-regulation of clusterin expression has been shown to protect renal tissue from apoptosis induced by several kinds of stimuli, including urinary tract obstruction and oxidative stress.[18–20] To our knowledge, however, the significance of clusterin expression in therapy-induced apoptosis in RCC has not been well characterized; therefore, clusterin cDNA was introduced into a human RCC cell line, ACHN, which scarcely expresses the *clusterin* gene, and the effects of clusterin overexpression on cytotoxic chemotherapy both *in vitro* and *in vivo* were investigated.

We initially demonstrated that overexpression of clusterin in ACHN cells confers no advantage on the proliferation of ACHN cells. However, clusterin-transfected ACHN cells were highly resistant to cisplatin treatment compared with control cells. In addition, treatment with a sublethal dose of cisplatin induced DNA fragmentation as well as cleavage of PARP protein in control cells, but not in clusterin-transfected cells. Consistent with *in vitro* studies, intravenous administration of cisplatin to nude mice bearing the ACHN tumors resulted in substantial growth inhibition of the control tumors compared with the clusterin-transfected tumors; that is, 60 days after tumor cell implantation, clusterin-transfected ACHN cells formed tumors more than twice as large as those formed by control cells. Taken together, these findings strongly suggest that the clusterin expression in human RCC cells contributes to the acquisition of resistance to cisplatin treatment, in part through inhibition of the apoptotic pathway.

We previously reported that clusterin could be a good therapeutic target for advanced prostate cancer using antisense oligodeoxynucleotide technology.[11–14] Moreover, Parczyk *et al.* reported that clusterin mRNA is overexpressed in RCC specimens compared with adjacent normal tissue. Considering these findings, it is suggested that the strategy of targeting the *clusterin* gene could be an attractive approach for advanced RCC; that is, if combined with antisense clusterin oligodeoxynucleotide therapy, the effi-

cacy of conventional treatment for RCC, including chemotherapy, radiation and immunotherapy, may potentially be enhanced.

In conclusion, the overexpression of clusterin in RCC cells causes acquisition of resistance to cisplatin both *in vitro* and *in vivo* through the inhibition of cisplatin-induced apoptosis. Although further investigation of the mechanism of drug resistance in advanced RCC is needed, our present findings strongly suggest that it will be valuable to develop a novel therapeutic strategy targeting the *clusterin* gene for patients with advanced RCC.

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