Enhanced Radiation Sensitivity in Prostate Cancer by Inhibition of the Cell Survival Protein Clusterin

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ABSTRACT

Purpose: The purpose of this study is to evaluate the role of the cell survival gene clusterin in radiation-induced cell death in human LNCaP and PC-3 prostate cancer models.

Experimental Design: Radiation sensitivities were compared in parental and clusterin-overexpressing LNCaP cells and in PC-3 cells and tumors treated with antisense or mismatch clusterin oligonucleotides.

Results: Clusterin-overexpressing LNCaP cells were less sensitive to irradiation with significantly lower cell death rates (23% after 8 Gy) compared with parental LNCaP cells (50% after 8 Gy) 3 days after irradiation. Clusterin expression in PC-3 cells after radiation was found to be up-regulated in a dose-dependent manner in vitro by 70% up to 12 Gy and in vivo by 84% up to 30 Gy. Inhibition of clusterin expression in PC-3 cells using antisense oligonucleotides (ASOs) occurred in a sequence- and dose-dependent manner and significantly enhanced radiation-induced apoptosis and decreased PC-3 cell growth rate and plating efficiency. Compared with mismatch control oligonucleotide treatment, clusterin ASO treatment enhanced radiation therapy and significantly reduced PC-3 tumor volume in vivo by 50% at 9 weeks. In addition, TUNEL staining revealed increased number of apoptotic cells in clusterin ASO-treated and irradiated PC-3 tumors, compared with treatment with mismatch control oligonucleotides plus radiation.

Conclusions: These findings support the hypothesis that clusterin acts as a cell survival protein that mediates radioresistance through the inhibition of apoptosis. In vivo results further suggest that inactivation of clusterin using ASO technology might offer a novel strategy to improve results of radiation therapy for prostate cancer patients.

INTRODUCTION

Prostate cancer is the most common cancer in North American men. Over 180,000 new cases and 31,400 deaths are expected for the year 2000 in the United States (1). A mainstay in the treatment of prostate cancer is the use of external beam radiation in localized disease. However, local recurrences and radioresistance remain significant clinical problems. For patients with high-risk localized prostate cancer, biochemical and clinical relapse after radiotherapy is significant, with >50% of patients having prostate-specific antigen failure within 5 years in most series (2). Survival has been improved with adjuvant androgen withdrawal therapy when used for 3 years (3). Many patients, however, are intolerant of long-term adjuvant therapy, may not have full recovery of testicular function, and recurrent disease still remains a significant problem.

Androgen resistance develops, in part, from up-regulation of survival or antiapoptotic genes after androgen withdrawal (4, 5), which is increasingly being used in combination with radiation therapy. Increased expression of survival or antiapoptotic-associated genes has been shown to confer resistance against a variety of apoptosis-inducing agents, including radiation, androgen-withdrawal therapy, and chemotherapy (6–8). In addition, clinical studies have also shown that increased expression of antiapoptotic genes in prostate cancer specimens were predictive of a poorer response to radiotherapy (9). Therefore, up-regulation of these cell survival genes may confer a broad spectrum of resistance given that the mechanism of cell death caused by androgen ablation, chemotherapy, and radiation is caused through the induction of similar or overlapping apoptotic pathways.

Clusterin, also known as testosterone-repressed prostate message 2 or sulfated glycoprotein 2, was first isolated from ram rete testes fluid (10) and has been implicated in a wide variety of physiological and pathological processes, including tissue remodeling, reproduction, lipid transport, membrane protection, complement defense, and apoptotic cell death (11). Because clusterin expression is increased in various benign and malignant tissues undergoing apoptosis, it has been associated with cell death (12–15). Recent observations show that clusterin acts in a chaperone-like manner similar to that of small heat shock proteins, potently inhibiting stress-induced protein precipitation in vitro and appears to improve cell survival in vivo (16). In prostate cancer, experimental and clinical studies suggest that clusterin expression is associated with androgen-independent recurrences and has a protective role against apoptotic cell death...
Clusterin levels increase up to 17-fold in radical prostatectomy specimens shortly after androgen withdrawal, consistent with its function in stress responses and inhibition of cell death (18).

Elucidation of the pathogenic role of candidate genes implicated in tumor progression is a rapidly progressing field of cancer research and has provided a steadily growing list of candidates. Known nucleotide sequences of cancer-relevant genes offer the possibility to design tailored anticancer agents that lack many of the toxic side effects displayed by conventional therapeutics. An ASO\(^3\) is designed to target the complementary sequence within a given RNA species, and once delivered into the target cell, hybridize with its RNA complement to inhibit translation of the disease-relevant protein. ASOs bind to a selected target mRNA by Watson-Crick base pairing, with subsequent inhibition of mRNA processing or translation by a variety of mechanisms, including prevention of mRNA transport, splicing, or translational arrest. Inhibition of gene expression is mainly accomplished by steric hindrance of the target mRNA at the ribosomal entry site and by recruitment of endogenous RNase H (19). Antisense therapy that targets the apoptotic rheostat in cells or interferes with signaling pathways involved in cell proliferation and growth are particularly promising in combination with conventional anticancer treatments. We have previously reported that inhibition of clusterin expression using sequence-specific ASOs enhance chemotherapy-mediated apoptosis in hormone refractory prostate cancer (17, 20), renal cell cancer (21), and urothelial cancer (22).

In this study, we investigated the role of clusterin on radiation sensitivity in prostate cancer tumor models. Using human LNCaP tumor cells stably transfected to overexpress clusterin (8), we evaluated the effect of clusterin overexpression on radiation response. Human PC-3 tumor cells, which intrinsically express clusterin, were then used to evaluate changes in cell proliferation and growth are particularly promising in combination with conventional anticancer treatments. We have previously reported that inhibition of clusterin expression using sequence-specific ASOs enhance chemotherapy-mediated apoptosis in hormone refractory prostate cancer (17, 20), renal cell cancer (21), and urothelial cancer (22).

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MATERIALS AND METHODS

Tumor Cell Lines. PC-3, derived from human prostate cancer, was purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD), supplemented with 5% heat-inactivated FCS.

LNCaP cells were kindly provided by Dr. Leland W. K. Chung (University of Virginia, Charlottesville, VA) and maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 5% heat-inactivated FCS. A cDNA probe was generated by reverse transcription-PCR from total RNA of human kidney using primers 5'-AAG-GAAATTCAGTTGCaAAA-3' (sense) and 5'-ACAGACTCCCCCGGAAAT-3' (antisense) for clusterin, and 5'-TGCTTTTACTCTCGTAAATGAT-3' (sense) and 5'-ATATTTGGCAGGTTTTTCTAG-3' (antisense) for GAPDH. Density of bands for clusterin was normalized against that of GAPDH by densitometric analysis.

MTT assay. The in vitro growth inhibitory effects of clusterin ASO plus irradiation on PC-3 cells were assessed using the MTT assay as described previously (18). 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 oligonucleotides at 500 nM for 2 days. After oligonucleotide treatment, cells were irradiated as described above. Every 24 h over a period of 5 days, MTT assays were carried out adding 20 µl of 5 mg/ml MTT (Sigma Chemical Co.) in PBS to each well, followed by incubation for 1 h 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 for the vehicle-treated cells to determine the percentage of survival. Each assay was performed in triplicate.

Clonogenic Assay. After irradiation with 4 Gy, PC-3 cells were trypsinized and resuspended in DMEM (Life Technologies, Inc.), supplemented with 5% heat-inactivated FCS. The same day, cell concentrations were determined by Coulter

\(^3\) The abbreviations used are: ASO, antisense oligonucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; XRT, X-ray therapy.
counting, and known numbers of cells were plated into Petri dishes containing 5% DMEM. Colonies >50 cells were counted after 14 days of growth. Plating efficiency was defined as number of colonies divided by the number of plated cells.

**Flow Cytometric Analysis.** PC-3 cells were plated on 100-mm dishes for 2 days until they were 50% confluent. Cells were then treated with 500 nm antisense or mismatch clusterin oligonucleotides once daily as described above. After 2 days of treatment, plates were irradiated with 4 Gy as described above. LNCaP cells were plated on 150-mm dishes until they were 70% confluent. Cells were then either irradiated with 8 Gy as described above or received a mock treatment for control. Three hours later, cells were trypsinized and resuspended in 150-mm dishes. After reaching confluency 4 days later, cells were trypsinized to produce a single cell suspension, centrifuged at 1500 rpm for 10 min, and fixed with 70% ethanol and recentrifuged. Cells were then incubated with RNase (0.5 mg/ml) at 37°C for 30 min, recentrifuged and stained with 1 ml of 50 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO), a fluorescent dye that intercalates double-stranded DNA. The DNA profile was then analyzed using a dual laser flow cytometer.

**In Vivo Treatments.** Approximately 1 × 10⁶ of PC-3 cells were inoculated s.c. with 0.1 ml of Matrigel (Becton Dickinson Labware, Bedford, MA) on the flank of 6–8-week-old male athymic mice under halothane anesthesia (5% induction and 1.5% maintenance concentration). When PC-3 tumors grew to 10 mm in diameter, usually 4–6 weeks after injection, mice were randomly selected for treatment with clusterin ASO plus radiation or mismatch control oligonucleotide plus radiation. Each experimental group consisted of 8 mice. After randomization, 12.5 mg/kg clusterin ASO or mismatch control oligonucleotide was injected i.p. once daily into each mouse for 21 days. Tumors received total irradiation of 15 Gy fractionated into three equal doses delivered on days 6, 9, and 12. Irradiation was performed with a Philips RT 250 X-ray machine at 25 kVp using a 0.5-mm copper filter. A special jig, comprised of a lead shell with an opening to allow for exposure of the tumor protruding from the back of the mouse, was used to provide whole body shielding. Assessment of tumor volume was followed up weekly over 10 weeks after irradiation. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

**TUNEL Staining.** A modified TUNEL technique (23) was used to detect apoptotic cells in PC-3 tumors using the ApopTag in Situ Apoptosis Detection System (Oncor, Gaithersburg, MD) according to the manufacturer’s protocol. The number of positively stained cells/high power field in five random fields was counted and averaged. PC-3 tumors were harvested for TUNEL-staining 1 day after completion of the same treatment schedule described above (day 22).

**RESULTS**

**Effect of Clusterin Overexpression on Radiation Sensitivity in LNCaP Cells.** Flow cytometry was used to demonstrate differences in growth arrest and apoptosis of parental (LNCaP/P) and clusterin-overexpressing (LNCaP/T) LNCaP cells after radiation (8 Gy). Three days after radiation, the proportion of cells undergoing apoptosis (sub G₁-G₀ fraction) was significantly lower in LNCaP/T (23%) compared with LNCaP/P (50%) cells ($P < 0.05$, based on differences of mean values after triplicate analysis using Student’s $t$ test). No significant differences in cell cycle distributions between these two cell lines could be seen before radiation (Table 1).

**Enhanced Expression of Clusterin after Radiation in PC-3 Cells and PC-3 Tumors.** Northern blot analysis was used to determine the effects of radiation on clusterin mRNA expression in PC-3 cells and tumors. As shown in Fig. 1, clusterin mRNA induction increased in a dose-dependent manner by radiation both in vitro and in vivo at radiation doses up to 12 Gy (in vitro) and 30 Gy (in vivo), respectively. Time course experiments in PC-3 cells demonstrated that clusterin mRNA up-regulation peaked by 72 h after radiation (Fig. 2).

| Effect of Combined Treatment with Clusterin ASO and Radiation on PC-3 Cell Growth Rate and Plating Efficiency. | Northern analysis confirms sequence-specific and dose-dependent antisense effects of the test oligonucleotide (Fig. 3). To determine the effects of combined treatment with ASO and radiation on clusterin levels, PC-3 cells were treated with either 500 nm antisense or a 2-base mismatch clusterin oligonucleotide once daily for 2 days. The next day, cells were irradiated with 4 Gy 1 h before media of all plates were renewed. Three days later, total RNA was extracted from cultured cells, and clusterin and GAPDH mRNA levels were analyzed by Northern blotting. Pretreatment of PC-3 cells with clusterin ASO decreased clusterin mRNA levels in nonirradiated cells by 66% and in irradiated cells by 46% in comparison to cells treated with mismatch control oligonucleotides (Fig. 4).

**Effect of Combined Treatment with Clusterin ASO and Radiation on PC-3 Cell Growth Rate and Plating Efficiency.** To determine whether antisense-induced inhibition of clusterin expression alters PC-3 cell survival after radiation, PC-3 cell growth rates after combined treatment with either 500 nm antisense or mismatch clusterin oligonucleotides (once daily for 2 days) and 4 Gy of radiation (1 day later) were examined using the MTT assay. As shown in Fig. 5A, ASO treatment significantly decreased cell viability by 4 days after radiation ($P < 0.05$). The same treatment schedule was used for clonogenic assays (Fig. 5B). Plating efficiency decreased in a dose-dependent manner ~6-fold after 500 nm clusterin ASO compared with mismatch control oligonucleotides ($P < 0.05$).
Effect of Combined Treatment with Clusterin ASO and Radiation on PC-3 Cell Growth Arrest and Apoptosis.

Flow cytometry was used to quantify changes in cell cycle and apoptosis 5 days after combined treatment with ASO and radiation (4 Gy). After the same treatment schedule described above, the fraction of cells undergoing growth arrest (G2-M block) or apoptosis (sub G1-G0 fraction) was significantly higher after treatment with clusterin ASO plus radiation (75%), compared with mismatch control oligonucleotides plus radiation (50%), treatment with clusterin ASO alone (39%), or mismatch control oligonucleotides alone (33%; Table 2). 

Enhanced Radiosensitivity of PC-3 Tumors after Treatment with Clusterin ASOs in Vivo. As shown in Fig. 6A, clusterin ASO significantly enhanced radiosensitivity of PC-3 tumors, reducing mean tumor volume >50% by 9 weeks after initiation of treatment (P < 0.05 by Student’s t test). In addition, TUNEL-staining detected a 2.5-fold increase in the numbers of apoptotic cells in PC-3 tumors treated with clusterin ASO plus radiation, compared with those treated with mismatch control oligonucleotides plus radiation (Fig. 6, B and C, respectively).
Inhibition of Clusterin Increases Radiosensitivity

Increased resistance to radiation (7). We hypothesize that adaptation of survival genes and tumor hypoxia (7, 26). Selective inhibition of expression of these genes would result in improved radiation sensitivity and thereby identify potential clinical therapeutic targets.

ASOs offer one strategy to functionally inhibit genes implicated in radiation resistance. ASOs specifically hybridize with complementary mRNA regions of a target gene and thereby inhibit gene expression by forming RNA/DNA duplexes (18, 27). Several ASOs targeted against specific genes involved in neoplastic progression have been evaluated as potential therapeutic agents (28–31), and the combined use of an ASO with cytotoxic chemotherapy and radiation have been demonstrated to produce more potent antineoplastic effects in several tumor models (32–38). We recently demonstrated that ASO-induced down-regulation of antiapoptotic genes such as bcl-2 (4, 31, 35) and clusterin (8, 17, 20, 21) significantly delayed progression to androgen independence after castration and enhanced chemosensitivity in several prostate cancer models. Like androgen ablation and chemotherapy, radiation is an apoptotic trigger that induces programmed cell death in subpopulations of tumor cells. Radiation may cause cell death through the induction of similar or overlapping apoptotic pathways, but little is known about the specific antiapoptotic genes and their contribution to radiation sensitivity in prostate cancer (7, 39).

The role of clusterin in apoptosis has been of some debate with recent data suggesting a proapoptotic rather than an antiapoptotic role after radiation in human MCF-7 breast cancer cells (40). Data reported herein, as well as in our previous studies (8, 17, 41), however, suggest that clusterin plays a protective role against apoptosis induced by various stimuli. The seemingly paradoxical roles for clusterin in apoptosis are perhaps analogous to those ascribed to two forms of Bcl-x that arise from alternative splicing (42–44). Clusterin can exist in several molecular forms, although they apparently arise from posttranslational modifications of a single mRNA transcript (45, 46).

In this study, clusterin overexpression significantly decreased the extent of radiation-induced apoptosis in LNCaP tumor cells, supporting a protective function of clusterin. In PC-3 cells and tumors, clusterin levels increased in response to even low doses of radiation. To clarify the functional role of the increased clusterin expression after radiation, we then tested whether clusterin inhibition using ASO technology would increase or decrease in radiosensitivity. Phosphorothioate clusterin ASO used in this study has been shown to inhibit clusterin expression in PC-3 cells in a dose-dependent and sequence-specific manner both in vitro and in vivo (17, 20–22). Northern blot analyses after combined treatment demonstrate that clusterin mRNA expression in PC-3 cells remains suppressed by ASO treatment even after radiation. Furthermore, when clusterin ASO was administered with radiation, PC-3 cell growth was significantly inhibited both in vitro and in vivo through increased cell cycle arrest and apoptosis. Pretreatment of PC-3 cells with clusterin ASO before radiation with 4 Gy reduced viability >30% and plating efficiency >6-fold. Consistent with these in vitro results, synergistic effects of combined use of clusterin ASO plus radiation was also observed in vivo. Systemic administration of clusterin ASO plus radiation suppressed PC-3 tumor growth by >50%, compared with treatment with

**DISCUSSION**

During the last few years, the implementation of three-dimensional conformal radiation techniques (24) and the use of temporary androgen withdrawal (3) have significantly improved long-term results of radiation therapy for locally advanced prostate cancer. However, limitations of these techniques include increased risk of local complications when radiation doses exceed 70 Gy (25) and side effects associated with androgen ablation. Furthermore, high local and systemic recurrence rates after combined hormone and radiation therapy illustrate that resistance and progression remain common events (3, 25). Therefore, novel combinatorial treatment strategies are required to further improve radiation sensitivity of prostate cancer.

Mechanisms of radiation resistance in prostate and other cancers remain poorly defined but include increases in expression of survival genes and tumor hypoxia (7, 26). Selective pressures of hypoxia, androgen withdrawal, and radiation may result in further increased expression of these survival genes. For example, overexpression of Bcl-2 in LNCaP cells resulted in increased resistance to radiation (7). We hypothesize that adaptive increases in antiapoptotic genes after radiotherapy increase cell survival and accelerate progression and emergence of a resistant phenotype. Selective inhibition of expression of these genes would result in improved radiation sensitivity and thereby identify potential clinical therapeutic targets.

Clusterin ASO, mismatch control oligonucleotides, or radiation (XRT) on clusterin mRNA expression of PC-3 cells in vitro. PC-3 cells were treated with either 500 nM antisense or 2-base mismatch clusterin oligonucleotides once daily for 2 days. The next day, cells were irradiated with 4 Gy 1 h before media of all plates were renewed. Three days later, total RNA was extracted from culture cells, and clusterin and GAPDH mRNA levels were analyzed by Northern blotting. Pretreatment of PC-3 cells with either 500 nM antisense or 2-base mismatch clusterin oligonucleotides decreased clusterin mRNA levels in nonirradiated cells by 68% and in irradiated cells by 46%. Clusterin mRNA levels were quantified (after normalization to GAPDH mRNA levels) by laser densitometry. Each column represents the mean value of triplicate analysis with SD. Numbers on the Y-axis symbolize arbitrary densitometric units.

Fig. 4 Effects of single and combined treatment with either clusterin ASO, mismatch control oligonucleotides, or radiation (XRT) on clusterin mRNA expression of PC-3 cells in vitro. PC-3 cells were treated with either 500 nM antisense or 2-base mismatch clusterin oligonucleotides once daily for 2 days. The next day, cells were irradiated with 4 Gy 1 h before media of all plates were renewed. Three days later, total RNA was extracted from culture cells, and clusterin and GAPDH mRNA levels were analyzed by Northern blotting. Pretreatment of PC-3 cells with either 500 nM antisense or 2-base mismatch clusterin oligonucleotides decreased clusterin mRNA levels in nonirradiated cells by 68% and in irradiated cells by 46%. Clusterin mRNA levels were quantified (after normalization to GAPDH mRNA levels) by laser densitometry. Each column represents the mean value of triplicate analysis with SD. Numbers on the Y-axis symbolize arbitrary densitometric units.

Inhibition of Clusterin Increases Radiosensitivity
mismatch control oligonucleotides plus radiation. We previously reported that clusterin ASO treatment alone does not inhibit tumor progression; however, when used in combination with other cell death signals like androgen ablation or chemotherapy, treatment responses were synergistically enhanced (8, 17, 20–22). The relative increase of the number of apoptotic cells after combined ASO treatment and radiation detected by TUNEL staining in PC-3 tumors suggests that delayed tumor progression.

Fig. 5  Effect of combined treatment with antisense or mismatch clusterin oligonucleotides plus radiation (XRT) on PC-3 cell growth rate and plating efficiency (in vitro). A, PC-3 cells were treated with either 500 nM antisense or a 2-base mismatch clusterin oligonucleotide once daily for 2 days, whereas control group (radiation only) did not receive any oligonucleotide treatment. The next day, all cells were irradiated with 4 Gy. Over 5 consecutive days, cell viability was determined by the MTT assay. Each data point represents the mean of triplicate analysis with SD. *, differs from control by Student’s t test (P < 0.05). B, PC-3 cells were treated with either 100 or 500 nM antisense or mismatch clusterin oligonucleotides once daily for 2 days, whereas control group (radiation only) did not receive any oligonucleotide treatment. After irradiation with 4 Gy, PC-3 cells were trypsinized and resuspended in 5% DMEM. Colonies > 50 cells were counted after 14 days of growth. Plating efficiency (number of colonies/number of plated cells) decreased in a dose-dependent manner after treatment with ASO, up to 6-fold by 500 nM clusterin ASO compared with mismatch control oligonucleotides. C, PC-3 cells were treated as described in 5B above. A dose dependent increase in radiation sensitivity is observed using the MTT assay. Each column means number of cells after triplicate analysis.

| Table 2  Effect of clusterin ASO treatment and radiation (4 Gy) on the cell cycle distribution of PC-3 cells |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Sub G1-G0 | G1-G0 | S | G2-M | SD  |
| Clusterin ASO   | 15%        | 43%    | 18%    | 24%    | ±2%  |
| Mismatch control| 13%        | 44%    | 23%    | 20%    | ±4%  |
| Clusterin ASO + radiation | 54%       | 11%    | 14%    | 21%    | ±4%  |
| Mismatch control + radiation | 36%       | 28%    | 18%    | 14%    | ±4%  |

* Values represent the mean of triplicate analysis ± SD.
* P < 0.05, different from mismatch control + radiation by Student’s t test.
progression with combined clusterin ASO plus radiation results from enhanced radiation-induced apoptosis. These results demonstrate for the first time that targeted suppression of clusterin increases the radiosensitivity of PC-3 cells both in vitro and in vivo.

Collectively, the findings of this study in the LNCaP and the PC-3 tumor models identify clusterin as an inhibitor of radiation-induced apoptosis and add additional support to the hypothesis that clusterin up-regulation after apoptotic signals represents an adaptive cell survival mechanism after a variety of apoptosis-inducing stimuli. The preclinical data presented here provide proof of principle support for designing clinical studies with combined antisense clusterin plus radiation therapy for patients with organ-confined or locally advanced prostate cancer.

ACKNOWLEDGMENTS

We thank Mary Bowden and Howard Tearle for their excellent technical assistance, as well as Dr. Lawrence Mayer and Visia Dragowska (Department of Molecular Sciences, British Columbia Cancer Agency, Vancouver, Canada) for their support and technical assistance in flow cytometry.

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