Silencing Expression of the Clusterin/Apolipoprotein J Gene in Human Cancer Cells Using Small Interfering RNA Induces Spontaneous Apoptosis, Reduced Growth Ability, and Cell Sensitization to Genotoxic and Oxidative Stress

Ioannis P. Trougakos, Alan So, Burkhard Jansen, Martin E. Gleave, and Efstatios S. Gonos

ABSTRACT
Clusterin/Apolipoprotein J (CLU) is a heterodimeric ubiquitously expressed secreted glycoprotein that is implicated in several physiological processes and is differentially expressed in many severe physiological disturbances, including tumor formation and in vivo cancer progression. Despite extensive efforts, clarification of CLU’s biological role has been exceptionally difficult and its precise function remains elusive. Short RNA duplexes, referred to as small interfering RNAs (siRNAs), provide a new approach for the elucidation of gene function in human cells. Here, we describe siRNA-mediated CLU gene silencing in osteosarcoma and prostate human cancer cells and illustrate that CLU mRNA is amenable to siRNA-mediated degradation. Our data demonstrate that CLU knockdown in human cancer cells induces significant reduction of cellular growth and higher rates of spontaneous endogenous apoptosis. Moreover, CLU knockdown cancer cells were significantly sensitized to both genotoxic and oxidative stress induced by chemotherapeutic drugs and H2O2, respectively. These effects were more pronounced in cell lines that express high endogenous steady-state levels of the CLU protein and occur through hyperactivation of the cellular apoptotic machinery. Overall, our results reveal that, in the distinct cellular contexts of the osteosarcoma and prostate cancer cells assayed, CLU is a central molecule in cell homeostasis that exerts a cytoprotective function. The described CLU-specific siRNA oligonucleotides that can potently silence CLU gene expression may thus prove valuable agents during antitumor therapy or at other pathological conditions where CLU has been implicated.

INTRODUCTION
Clusterin/Apolipoprotein J (CLU) in humans is constitutively secreted by several cell types, and it is found in all human body fluids analyzed thus far (1). In human plasma, CLU exists in high-density lipoprotein particles that contain apolipoprotein A-I and cholesteryl ester transfer protein (2). Nevertheless, CLU, apart from functioning as an apolipoprotein, is also implicated in additional intra- or extracellular processes. The primary translation product of the CLU gene is a polypeptide of 449 amino acids, where the first 22 amino acids represent the classical hydrophobic secretory signal sequence. Maturation of the primary translation molecule include disulfide bonding, conversion to a high-mannose endoplasmic reticulum-associated form of ~60 kDa, extensive additional N-linked glycozylation, and finally, proteolytic cleavage in the trans-Golgi compartments that result in the mature secreted heterodimeric CLU protein form of ~70–80 kDa (3). Interestingly, another CLU protein form of ~55 kDa, designated as nuclear (n)-CLU55 (4), has been recently described in MCF-7 cells. According to a recent study (5), this protein form originates, after apoptosis induction, from a 49-kDa primary product (c-CLU49) that is translated from an alternatively spliced CLU transcript.

Many diverse physiological functions have been attributed to CLU, including tissue remodeling, membrane recycling, lipid transportation, cell-cell or cell-substratum interactions, and sperm maturation (6, 7). In addition, it was recently proposed that CLU functions as an extracellular chaperone that stabilizes stressed proteins in a folding-competent state (8). Another prominent feature of the CLU protein is its up-regulation in many severe physiological disturbance states, including kidney degenerative diseases and several neurodegenerative conditions (7, 9). CLU also exerts a significant role during tumorigenesis and progression of several human cancers. During in vivo progression to carcinogenesis, higher CLU levels were seen in prostate and kidney carcinomas when compared with normal tissues (10, 11), whereas CLU is also up-regulated in anaplastic large-cell lymphomas (12), in ovarian cancer (13), and during seminal vesicle carcinogenesis progression (14). Importantly, in breast tumor cells where epithelial normal cells were always negative for CLU expression, CLU protein accumulation correlates with the aggressiveness of a given breast tumor (15).

From all of the functions attributed to the CLU protein thus far, its implication in apoptosis has been primarily investigated. Despite extensive efforts, the role of CLU during apoptosis remains largely an enigma, the main cause being the intriguingly distinct and usually opposing functions proposed in an array of various cell types and tissues, including cancer cells (3) and CLU knockout mice (16, 17). According to some studies, CLU overexpression in various cell lines either could not confer any protection to apoptosis-inducing agents or exerts a proapoptotic function (4, 17–20). We have cloned CLU as a senescence-induced gene (21), and we have shown that CLU is also overexpressed in human diploid fibroblasts exposed to various types of stress (21–23), and it accumulates in the human serum during diabetes type II and atherosclerosis (24). Considering that CLU overexpression in HDFs protects cells from oxidative stress (23), its overexpression in senescent cells is unlikely to be related to a proapoptotic function (3). In addition, CLU gene overexpression in human androgen-dependent LNCaP prostate cells rendered them highly resistant to androgen ablation in vivo (25) and to treatment with chemotherapeutic agents (26), whereas antisense oligonucleotides targeting CLU resulted in significant increase of chemosensitivity in prostate cancer (27).

Given the direct involvement of the CLU protein in human carcinogenesis, as well as the fact that a therapeutic (OGX-011) inhibiting CLU is under development for treating human prostate cancer and other CLU-expressing malignancies (28), we sought to investigate the effect of altering CLU gene expression and to provide conclusive evidence regarding its function during apoptosis. We specifically knocked down the secreted (s)-CLU protein form in three osteosarcoma (OS) cell lines and in a prostate cancer cell line by using small interfering RNAs (siRNAs) and provide evidence that CLU knockdown induces growth retardation that is accompanied by higher rates of spontaneous endogenous apoptosis. Moreover, CLU knockdown...
resulted in significant sensitization to both genotoxic and oxidative stress-inducing agents. Our data suggests that cell sensitization may be directly related to activation of the cellular apoptotic machinery.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. OS cell lines (KH OS, Sa OS, and U-2 OS) and PC-3 cells were purchased from the American Tissue Culture Collection. OS cells were cultured in DMEM (Life Technologies, Inc., San Diego, CA) supplemented with 10% FCS, 2 mM glutamine, and 1% nonessential amino acids (complete medium) and PC-3 cells in DMEM supplemented with 5% heat-inactivated FCS.

Identification of Suitable CLU cDNA Sequences to be Targeted by siRNA–RNA Oligonucleotide Synthesis. The human CLU cDNA was scanned to identify sequences of the AA(N 19 )UU type that fulfill the required criteria for siRNA (29). Two such sequences were found 433 (Cl-I) and 664 (Cl-II) nucleotides downstream of the CLU gene transcription initiation codon. Two additional oligonucleotides used targeted a region 1620 nucleotides downstream of the CLU gene transcription initiation codon (Cl-III) and the transcription initiation site (Cl-V; Table I). BLAST analysis showed no homology with other known human genes. Selected RNA oligos were synthesized by Dharmacon Research, Inc. (Lafayette, CO). The Scramble-I (Sc-I) and Scramble-II (Sc-II) oligonucleotides used were purchased from Dharmacon.

siRNA Transfection. siRNA transfection of the Cl-I, Cl-II, and scrambled RNA duplexes in exponentially growing OS cells was performed as described previously (29, 30). Briefly, cells were seeded the day before siRNA transfection in 24-well plates containing 500 μl of complete medium and were ~40–50% confluent during transfection. For the transfection mixture, 100 nM siRNA duplex/well were used. The RNA duplex was diluted in Opti-MEM I (Life Technologies, Inc., San Diego, CA). When cells were treated with both Cl-I and Cl-II oligonucleotides, 100 nM of each siRNA duplex were used. Cell treatment with the siRNA oligonucleotides lasted for 2–3 days. In PC-3 cells, Lipofectin (Invitrogen) was used. PC-3 cells were treated with 10, 50, or 100 nM of the RNA duplexes after preincubation with 4 μg/ml Oligofectamine reagent in serum-free Opti-MEM I for 20 min. Four h after starting the incubation, the medium containing the RNA duplexes and Lipofectin was replaced with serum-free Opti-MEM I for 20 min. Apoptosis induction was then quantified by using the Oligofectamine reagent (Invitrogen) was used. PC-3 cells were treated with 10, 50, or 100 nM of the RNA duplexes after preincubation with 4 μg/ml Oligofectamine reagent in serum-free Opti-MEM I for 20 min. Four h after starting the incubation, the medium containing the RNA duplexes and Lipofectin was replaced with serum-free Opti-MEM I for 20 min. Apoptosis induction was then quantified by using the transfection medium to a final concentration of 0.35 μM, cells were incubated in the drug-containing transfection medium for 24 h, washed, allowed to recover for 72 h in complete medium, and counted.

Cell Proliferation-DNA Synthesis Analysis. For the quantitative determination of the DNA synthesis rate, the Cell Proliferation ELISA BrdUrd colorimetric immunoassay method (Roche, Inc., Basel, Switzerland) was used. Briefly, 5 × 10³ cells were seeded in sextuplicates in 96-well plates and treated with either the Sc-I siRNA oligonucleotide or the Cl-I CLU-specific oligonucleotide for 60 h. Cells were then labeled for 3 h with bromodeoxyuridine, and DNA synthesis was measured by using the ELISA BrdUrd assay according to the manufacturer’s instructions.

Quantitative Analysis of Endogenous Cellular Apoptosis or Apoptosis after Cell Treatment with DXR or H 2 O 2 -Cell Death Detection by Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) Assay. To assay the endogenous spontaneous cellular apoptosis, cells were treated with either the Sc-I or the Cl-I siRNA duplexes for 60 h. Subsequently, the cytoplasmic histone-associated DNA fragments, which are indicative of ongoing apoptosis, were quantitatively measured by using the Cell Death Detection ELISAPLUS photometric enzyme-immunoassay method (Roche, Inc.) according to the manufacturer’s instructions.

To assay cell sensitization and the extent of apoptosis induction after DNA damage or oxidative stress, an equal number of siRNA-treated cells were subcultured in complete medium containing either: (a) 0.35 or 1 μM DXR for 24 h or (b) 400 μM H 2 O 2 for 3 h followed by a recovery period in complete medium for 21 h. Apoptosis induction was then quantified by using the ELISAPLUS death assay.

to detect DNA fragmentation in cell monolayers after DXR treatment,
TUNEL was performed by using the in situ Cell Death Detection kit (Roche, Inc.) that relies on fluorescent labeling of DNA strand breaks. Briefly, equal cell numbers were seeded in duplicates in 6-well plates, allowed to attach overnight on coverslips, and treated with 0.35 or 1 μM DXR for 24 h. Subsequently, cells were either washed and DNA fragmentation was detected by TUNEL according to the manufacturer’s instructions or allowed to grow for additional 24 h in drug-free medium before the application of the TUNEL reaction. Positive apoptotic nuclei were recorded by fluorescence microscopy.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay. The combined effects of siRNA plus Paclitaxel (Sigma) on PC-3 cells were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (27). Briefly, 3 × 10^5 cells were plated in sextuplicates in 96-well plates, allowed to attach overnight, treated with 50 nM of either Cl-III, or CI-V or Sc-I control oligonucleotides for 4 h and allowed to recover for 48 h. Cells were then exposed to various concentrations of Paclitaxel, ranging from 0 to 500 nM, for 48 h before assaying cell survival with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

RESULTS

OS Cells as a Model System to Study CLU Function in Human Cancer Cells. We hypothesized that the diverse functions attributed to CLU during apoptosis in different cellular systems and tissues could reflect specific intrinsic properties of the cell lines used. Therefore, an ideal in vitro model system to study CLU function should fulfill the following criteria. Firstly, the selected cell lines should be closely related and they should preferentially originate from the same tissue. Secondly, they should be genetically well characterized and they should express different steady-state endogenous CLU protein levels. Finally, in the selected cell lines, CLU protein should accumulate at a distinct rate after cell exposure to apoptosis-inducing agents.

After a survey of several human cell lines and subsequent analysis of the various CLU protein forms in these cells, we concluded that three OS cell lines, namely U-2 OS, KH OS, and Sa OS, fulfill these criteria. As it can be seen in Fig. 1A, the selected OS cell lines express different endogenous CLU protein amounts, with the U-2 OS being the cell line with the highest CLU protein content (Fig. 1A, top panel). Moreover, these cells are well characterized regarding their genetic background because for instance the p53 gene is deleted in Sa OS cells, mutated in KH OS, but it is wild type in U-2 OS cells (Ref. 31; Fig. 1A, middle panel). Finally, we found that the steady endogenous CLU protein levels negatively correlate with the intensity of CLU protein accumulation after cell exposure to various DNA damage-inducing agents (Fig. 1B). The highest rate of CLU accumulation after cell exposure to DXR was found in KH OS cells that express the lowest endogenous CLU protein levels. In contrast, a moderate induction (up to 2-fold) of the CLU protein can be seen in the U-2 OS cells that express high endogenous levels of the CLU protein.

Efficient Silencing of the CLU Gene Expression in OS Cells by Using siRNA. Treatment of the three OS cell lines with the Cl-I or the Cl-II siRNA oligonucleotides was quite effective and resulted in significant knockdown of the cellular CLU protein levels (Fig. 2A–2C). The Cl-I oligonucleotide appeared to be slightly more effective than Cl-II in silencing the CLU gene. No CLU gene silencing was seen in the presence of the control Sc-I or the Sc-II (data not shown) oligonucleotides or in the absence of RNA duplexes from the transfection medium.

Next we addressed the issue whether the CLU-specific siRNA oligonucleotides could also inhibit the accumulation of the CLU protein after cellular exposure to DXR. As shown in Fig. 2, D and E, exposure of the KH OS and U-2 OS cells to DXR for 24 h in the presence of the Cl-I, Cl-II or a mixture of both the Cl-I, Cl-II oligonucleotides effectively abolished CLU protein accumulation. It is thus evident that in the presence of the Cl-I or the Cl-II oligonucleotides, the cellular CLU protein cannot be induced after cell exposure to apoptosis-inducing agents.

Phenotypic Effects in OS Cells after the Silencing of CLU Gene Expression by siRNA. The effects of silencing of the CLU gene expression in OS cells were studied by direct counting of the cells following siRNA by recording both cellular morphology and phenotype, as well as by clonogenic assays. CLU knockdown in KH OS and Sa OS cells did not result in any visible change on phenotype. However, the CLU knock down cells were found to be significantly growth retarded as compared with their control counterparts (Fig. 3A). In contrast at the U-2 OS cells, which express higher endogenous amounts of the CLU protein, the effects of CLU knockdown were more pronounced. U-2 OS-treated cells lost their firm adherence to plastic and acquired a rounding morphology (Fig. 3B). This phenotype was accompanied by severe growth retardation (Fig. 3A). To study whether a combination of both Cl-I and Cl-II RNA duplexes would be more effective in inhibiting cell growth, we treated cells with both these oligonucleotides. As shown in Fig. 3A for the KH OS and U-2 OS cells, only a slight increase in growth retardation was observed as compared with the Cl-I-treated cells. Finally, to distinguish between the cytostatic and cytotoxic effects of CLU protein elimination, we directly assayed CLU knock down KH OS and U-2 OS cells for DNA synthesis and spontaneous endogenous apoptosis. CLU knock down cells showed a reduced DNA synthesis rate (Fig. 3C) and higher levels of spontaneous endogenous apoptosis (Fig. 3D) as compared with their sibling controls. Effects were again more pronounced in U-2 OS cells. In summary, these results suggest that the reduced number of CLU knock down cells is due to both a reduced rate of cell proliferation and an increased level of spontaneous apoptosis.

As it was reported recently that transfection of siRNAs or DNA vectors that express shRNAs from RNA Pol III promoters can trigger an IFN response (32, 33), we assayed our Sa OS and U-2 OS siRNA-transfected cells lines for the expression levels of a number of IFN-type I and type II genes. These include the type I viral-stimulated
proteins OAS-1, Stat1, and phosphorylated double-stranded RNA-dependent protein kinase as well as the type II β-subunits of the 20S immunoproteasome LMP7 and MECL-I (34, 35). As can been seen in Fig. 3E, none of the IFN-type I or -type II-inducible genes were up-regulated. Similarly, no activation of the IFN-type I pathway was found in four normal human cell lines after prolonged exposure to siRNA duplexes (see supplementary figure). These results are consistent with the reported genome-wide expression-profiling studies (36, 37) and with the observation that in human lung fibroblasts, although DNA vectors that express shRNAs can trigger the IFN response, transfection of the same cells with chemically synthesized siRNA duplexes corresponding to the shRNAs species produced by the vectors, although reduced the level of the target gene by ~80%, did not induce the IFN system (32). Conclusively, the phenotypic effects of CLU knockdown in OS cells are highly specific and are not related to the activation of any off-target IFN-inducible gene. As recent studies have demonstrated that gene silencing is sustained for more than seven cellular doublings (29), the effect of CLU knockdown in plating efficiency and growth after siRNA was subsequently studied by clonogenic assays. KH OS and U-2 OS cells were selected for these assays because they represent two extreme opposite cases as far as the endogenous CLU amount and the intensity of CLU accumulation during stress are concerned. CLU knock down KH OS cells when plated were firmly attached to the plastic (>90%) and only a few of the attached cells showed an abnormal morphology. However, the growth potential of the adherent cells was impaired as found 5 days postplating after analyzing the total colony number and size of the formed colonies (Fig. 4A–4A). CLU knock down U-2 OS cells were poorly attached to the plastic after trypsinization (~70%), and

![Fig. 2 A–C](image)

**Fig. 2.** A–C, effective silencing of the clusterin (CLU) gene expression in OS cells after small interfering RNA (siRNA) treatment for 60 h as revealed by whole-cell lystate immunoblotting analysis (reducing conditions) of the CLU protein endogenous levels in Sa OS (A), KH OS (B), and U-2 OS (C) cells. D, confocal laser scanning microscopy after CLU immunofluorescence localization in U-2 OS cells treated for 48 h with siRNA. Cl-I or Cl-II siRNA-treated cells show minimal levels of immunofluorescence. D and E, whole-cell lystate immunoblotting analysis of CLU protein levels in KH OS (D) and U-2 OS (E) cells after siRNA for 70 h and subsequent treatment with 0.35 µM doxorubicin (DXR) for 24 h in the presence of the indicated siRNA oligonucleotides. In the presence of the specific CLU siRNA oligonucleotides, the DXR-treated OS cells cannot accumulate the CLU protein. Con-I, mock-transfected cells; Sc-I, cells treated with the Scramble-I oligonucleotide; Cl-I, Cl-I cells treated with the CLU oligonucleotides Cl-I or Cl-I, respectively; Cl-I/II cells treated with a mixture of both Cl-I and Cl-II oligonucleotides; secreted (s)-CLU, the α-, β- chains (~40 kDa) of the secreted CLU protein form after reduction of the mature protein; cytoplasmic (c)-CLU, the intracellular did not reduced-precursor CLU protein form of ~60 kDa. Protein loading was verified by actin (bottom panels in A, B, C, D, and E). Molecular weight markers are indicated on the right of each blot. All assays were repeated at least three times. Bars in E, 10 µm.

![Fig. 3 A–E](image)

**Fig. 3.** A, relative growth rate of Sa OS, KH OS, and U-2 OS cells after small interfering RNA (siRNA)-mediated clusterin (CLU) gene expression silencing. Cells were treated with the indicated siRNAs for 70 h, and the total cell number was counted. A significant reduction of the cell number that is more pronounced in the U-2 OS cells can be seen in all CLU knocked down cells. Only a slight enhancement in growth retardation is observed when a combination of Cl-I and Cl-II oligonucleotides (Cl-I/II) was used. Growth of mock-transfected (Con-I) cells was set at 100%. B, morphological alterations in U-2 OS cells treated with the indicated siRNA oligonucleotides for three days. Note the rounding and shrinkage observed in the CLU knock down cells (arrow). C and D, endogenous DNA synthesis levels and spontaneous apoptosis in CLU knock down KH OS and U-2 OS cells, as estimated by using a Cell Proliferation ELISA BrdUrd colorimetric immunoassay (C) and a Cell Death Detection ELISA photometric enzyme-immunoassay (D), respectively. CLU knockdown is accompanied by reduced DNA synthesis (C) and an enhanced rate of endogenous spontaneous cellular apoptosis (D) in both cell lines. The effects are significantly pronounced in U-2 OS that express higher endogenous CLU levels. E, CLU knockdown by chemically synthesized siRNA duplexes does not induce an IFN response in OS cells. Sa OS and U-2 OS cells were treated with 100 nM of the indicated siRNA duplexes for 48 h. Subsequent immunoblotting analysis revealed that the presence of the siRNAs studied did not induce IFN type I (OAS-1, Stat1, and P-PKR) or type II (LMP7 and MECL-1)–stimulated genes. All assays were performed in triplicates and have been repeated two (C–E) or three (A) times. Each data point represents the mean of the independent experiments and bars denote SD. * indicates differences from their respective controls at P < 0.01; n.a., not assayed; abbreviations are as in Fig. 2. Bars in B, 15 µm.

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most of the adherent cells appeared quite abnormal in shape. Cells showed an extremely low proliferation potential (Fig. 4B1), and after 9 days in culture only, some small colonies could be seen (Fig. 4B2).

Sustained Silencing of CLU Gene Expression in OS Cells by siRNA Results in Significant Sensitization to Apoptosis Induced by Genotoxic and Oxidative Stress. Before CLU functional assays, we analyzed the DXR effects in OS cells because the drug-related reported effects vary in different cell types (38). As the DXR plasma concentration in treated patients falls into a range of 1–2 μM and decline into a range of 0.025–0.25 μM within 1 h (39), cells were treated with 0.35 and 1 μM of DXR. To analyze the extent of the DXR-mediated cell death, we scored apoptosis by TUNEL. A representative analysis in KH OS cells is shown in Fig. 5A. Attached cells after drug treatment for 24 h underwent significant morphological changes as compared with nontreated control cells (Fig. 5A, a1). At this time, cells exhibit an enlarged and flattened morphology that is reminiscent of senescence, whereas a significant number of them are TUNEL positive (Fig. 5A, a2 and a3). Ongoing apoptosis is also apparent 24 h later (Fig. 5A, a2 and a3), verifying that apoptosis is a dynamic process that continues even after DXR removal from the medium. In agreement with the results obtained by TUNEL, DXR treatment was accompanied by poly(ADP-ribose) polymerase cleavage; the antiapoptotic protein bcl-2 showed no altered expression in drug-treated KH OS and U-2 OS cells (Fig. 5B). After DXR treatment, accumulation of p53 protein and its downstream effectors related to either growth arrest (p21) or apoptosis (bax) was found only in U-2 OS cells, indicating that the cytostatic and cytotoxic effects mediated by DXR in OS cell lines rely on both p53-dependent and p53-independent mechanisms.

Next, we followed two complementary approaches to study the effect of CLU knockdown in OS cells exposed to DXR (Fig. 5, C1 and C2). siRNA-treated cells were either replated in complete medium and allowed to recover in complete medium for 72 h, and counted. The combined chemotherapy drug treatment and CLU siRNA is more effective in promoting OS cells death. Survival of control cells (Con-I) was set at 100%. D1 and D2, enrichment of the cyttoplasmic histone-associated-DNA fragments that are indicative of an ongoing apoptosis in KH OS (D1) and U-2 OS (D2) cells after siRNA with the indicated siRNA oligonucleotides for 60 h and postcultivation in siRNA-free medium containing 0.35 or 1 μM DXR for 24 h. E1 and E2, enrichment of the cyttoplasmic histone-associated-DNA fragments in KH OS (E1) and U-2 OS (E2) cells after siRNA with the indicated siRNA oligonucleotides for 60 h, exposure to 400 μM H2O2 in a siRNA-free medium for 3 h, and postcultivation in normal complete medium for 21 h. CLU protein knock down results in significant cell sensitization to both genotoxic and oxidative stress. Samples were analyzed in duplicates, and data points represent the mean of three independent experiments; bars denote SD; * indicates differences from their respective controls at P < 0.01 and ** differences from dark bars (in C1 and C2) at P < 0.05. Abbreviations are as in Fig. 2. Bars in (a1–a3), 20 μm.
were subsequently exposed to 0.35 \mu M DXR for 24 h, or they were exposed to 0.35 \mu M DXR in the presence of the CI-II or CI-I oligonucleotides. In both cases, viable cells were counted 3 days after DXR treatment. CLU knockdown KO cells appeared more sensitive to DXR than their control counterparts (Fig. 5C1,17), whereas DXR treatment appeared significantly more effective when it was combined with the presence of the CLU-specific siRNA oligonucleotides in the medium (Fig. 5C1,17). Similarly, CLU knockdown Sa OS cells were more sensitive to the DXR treatment (data not shown). In U-2 OS cells, both strategies appeared very effective, and CLU knockdown cells were significantly more sensitive to the drug as compared with controls (Fig. 5C2). When DXR treatment was performed in the presence of the CLU-specific siRNA oligonucleotides, the CLU knocked down cells were significantly more sensitive to the drug (Fig. 5C2), and massive apoptosis was observed. Finally, when U-2 OS cells were treated with both the CI-I and CI-II oligonucleotides, cells were almost eliminated (data not shown).

To understand the mechanism of cell sensitization after CLU knockdown, we directly assayed the intensity of apoptosis induction right after cell exposure to agents inducing genotoxic (DXR) or oxidative stress (H2O2). As shown in Fig. 5, D and E, exposure of the CLU knock down cells to either DXR or H2O2 resulted in a significantly higher rate of apoptosis in both KO OS and U-2 OS cells. This observation suggests that CLU directly affects or interacts with the cellular machinery involved in apoptosis by providing cytoprotective signals.

OS Cells Sensitization to Genotoxic and Oxidative Stress Because of CLU Gene Silencing Is Related to Activation of the Cellular Apoptotic Machinery. By analyzing the expression levels of other recently identified CLU protein forms, to our surprise, we found that the CI-I and CI-II oligonucleotides did not exert any significant effect on the putative 55 kDa (n-CLU55) CLU protein form in both KO OS and U-2 OS cells; only a minor effect on the 49 kDa s-CLU49 protein form level was detected (Fig. 6, A and B), although the binding sites of the CI-I or the CI-II oligonucleotides are common between the s-CLU and n-CLU mRNAs (5). We assume that the explanation of this effect relies on our observation that the n-CLU protein is extremely stable (unpublished data). Thus, CI-I and CI-II oligonucleotides specifically knocked down the s-CLU protein form.

We then assayed the expression levels of several proteins involved in regulating apoptosis in human cells. As it can be seen in Fig. 6, A and B, CLU knockdown in both KO OS and U-2 OS cells resulted in the down-regulation of the antiapoptotic molecule bcl-2. No effect was detected on levels of Ku70, a protein implicated in DNA damage repair and signaling that, moreover, binds n-CLU (4). Interestingly, in U-2 OS cells, which bear a functional p53 molecule, CLU knockdown apart from bcl-2 down-regulation is also accompanied by p53 accumulation and up-regulation of both its downstream proapoptotic effector, bax, and the cyclin-dependent kinase inhibitor, p21 (Fig. 6B). Moreover, CLU knock down U-2 OS cells when exposed to DXR showed a more intense and robust accumulation of the p53 protein (Fig. 6C) as compared with the Sc-I-treated cells. We suggest that sensitization of OS cells after CLU knockdown largely depends on the activation of the cellular proapoptotic machinery.

Effects of CLU Gene Silencing in PC-3 Prostate Cancer Cells. Having established the significant effects of CLU knockdown in OS cells, we then applied CLU siRNA in PC-3 human prostate cancer cells. PC-3 cells are p53 null (40) and express relatively low endogenous amounts of the s-CLU protein form similar to the Sa OS cells. In these cells, apart from using the CI-I and CI-II oligonucleotides, we also tested two additional CLU-specific siRNA oligonucleotides (Cl-III, CI-V; CI-V targets the s-CLU transcription initiation site). Usage of the CI-I and CI-II siRNA oligonucleotides in PC-3 cells resulted in similar effects to those described for OS cell lines (data not shown). As can be been seen in Fig. 7A–C, both the CI-III and CI-V oligonucleotides are quite effective in silencing CLU mRNA and protein expression in a sequence-dependent but dose-independent manner. More specifically, treatment of the PC-3 cells, for 1 day, with 10, 50, and 100 nm of either CI-III or CI-V siRNA oligonucleotides severely reduced CLU mRNA levels ranging from 60% to 98% (Fig. 7, A and B). This effect on mRNA levels was also evident at the protein level (Fig. 7C). Additionally and in agreement with findings in the U-2 OS cells, CLU knockdown in PC-3 cells resulted in significant morphological changes that resembled an ongoing apoptosis (Fig. 7D).

To determine whether treatment of PC-3 cells with CLU siRNA oligonucleotides could enhance the cytotoxic effects of chemotherapeutic drugs, PC-3 cells were treated first with the CI-III, CI-V or the Sc-I siRNA oligonucleotides and then incubated with medium containing various concentrations of paclitaxel for 2 days. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were then performed to determine cell viability. As shown in Fig. 8, CLU siRNA treatment significantly enhanced chemosensitivity of paclitaxel in a dose-dependent manner reducing the IC50 (the concentration that reduces cell viability by 50%) of paclitaxel by >90%, whereas the scrambled siRNA had no effect.

**DISCUSSION**

CLU is an enigmatic molecule, and despite extensive studies, it remains unclear whether its functions reflect multifunctionality or alternatively they mask a common function. In particular, CLU’s implication in cell death is still debatable because according to the relatively few studies where the effects of experimental manipulation of CLU expression on cell death and survival were analyzed, CLU reportedly relates to both a proapoptotic and an antiapoptotic function (see “Introduction”). The ability of 21-nt duplexes of siRNA to direct sequence-specific degradation of mRNA (29, 30) and thereby induce specific gene silencing in mammalian cells has raised the possibility that siRNA can be used to investigate gene function in a high-throughput fashion or to modulate gene expression in human diseases. Our presented results clearly dem-
onstrate that CLU mRNA is amenable to specific siRNA-induced degradation in vitro and that the secreted CLU protein form is an essential molecule for the cellular homeostasis of the OS and prostate cancer cells assayed. Moreover, it is evident that the primary function of CLU in all distinct genetic backgrounds of these cancer cells is antiapoptotic. The effects of CLU gene expression silencing are mainly related to the endogenous CLU protein levels expressed by a given cell line and are particularly highlighted in U-2 OS that express the highest CLU endogenous amounts among the cell lines assayed in this study. In U-2 OS cells, CLU knockdown resulted in significant growth retardation, higher rates of endogenous apoptosis, decreased plating efficiency (that may indicate a reduced metastatic potential), and significant sensitization to apoptosis-inducing agents. Interestingly, it was recently shown that CLU over-expression into human renal cell carcinoma cells enhances their metastatic potential (41). The proposed essential role of CLU for cellular homeostasis is clearly supported by the fact that CLU is associated with cells surviving programmed cell death during development (42, 43) and, in addition, it is highly expressed in an array of specialized cell types of adult human tissues and almost in all epithelial boundary cells and several nonepithelial secretory cell types (44). In agreement with the presented data, we previously demonstrated a cytoprotective role of the secreted CLU protein form in HDFs during cellular senescence (22, 23). Moreover, we have found that CLU antisense oligonucleotides chemosensitize human PC-3 prostate cancer cells (27), bladder cancer cells (45) and human renal cell CAKI-2 tumors (46) both in vitro and in vivo and showed that CLU overexpression in prostate cancer xenograft models results in acquisition of a chemoresistant phenotype (26). Similarly, according to a series of studies in CLU\textsuperscript{−/−} mice, it has been shown that: (a) CLU has anti-inflammatory properties and limits the severity of murine autoimmune myocarditis (47); (b) protects against ischemic brain damage and reduces postischemic cell mortality in the brain (16); (c) has a protective effect against heat-shock-mediated apoptosis (48); and (d) exerts a protective role against chronic glomerular kidney disease (49). Interestingly, no effects have been previously reported on cellular growth, DNA synthesis or spontaneous endogenous apoptosis in CLU\textsuperscript{−/−} mouse embryonic fibroblasts (16, 17) or in human cancer cells where CLU levels were reduced by using antisense oligonucleotides (27). Our unpublished comparative analysis shows that siRNA treatment is significantly more effective in knocking down the CLU protein levels than antisense oligonucleotides. Regarding differences between analysis in CLU\textsuperscript{−/−} mouse embryonic fibroblasts and our in vitro data, it is plausible CLU knockdown to result distinct phenotypes in normal and in cancer cells. Additionally, considering that CLU overexpression in SV40-immortalized human prostate epithelial cells slows down cell cycle progression, thus suggesting that CLU is a putative tumor suppressor gene (50), whereas CLU exhibits elevated levels during human colon cancer (51), it appears that only certain types of cancer cells have acquired dependence on CLU to survive and proliferate.

Our data indicate that CLU may also exert a significant function intracellularly. Apart from the exclusively intracellularly localized CLU precursor high mannose form of \textsim{60} kDa, in the rat ventral prostate model, various CLU cytoplasmic protein-forms were found to be related either to surviving or to dying tissue (19). Additionally, tumor necrosis factor \textalpha treatment of L929 cells induces the appearance of two novel cytoplasmic CLU forms exerting a cytoprotective function (52). In contrast, it was shown recently that certain intracel-

Fig. 7. Sequence-specific clusterin (CLU) gene silencing by siRNA in PC-3 tumor cells. A, PC-3 cells were treated with the shown concentrations of the indicated siRNA oligonucleotides for 1 day. CLU and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were then assayed by RNA blot analysis. B, quantitative analysis of the CLU mRNA levels shown in (A) after normalization to GAPDH mRNA levels. Each point represents the mean of triplicate analyses; bars represent SD; * differ from control at \textit{p} < 0.001. C, immunoblotting analysis (reducing conditions) of the secreted (s)-CLU and cytoplasmic (c)-CLU protein forms levels in cells treated with the indicated siRNA oligonucleotides as in (A); CLU siRNA is PC-3 cells is particularly effective. Vinculin immunoblotting was used to estimate protein loading (bottom panels). D, morphological changes two days after a daily treatment with \textsim{20} nM of the indicated siRNA oligonucleotides. Note the rounding and shrinkage of PC-3 cells treated with the CI-V oligonucleotide (arrows) that are morphological changes suggestive of apoptosis. CI-III, CI-V cells treated with the CI-III or the CI-V CLU siRNA oligonucleotides, respectively; abbreviations are as in Fig. 2; bars in D, \textsim{30} \mu M.

Fig. 8. Effect of paclitaxel treatment on clusterin (CLU) knock down PC-3 cells growth and apoptosis. Cells were treated with \textsim{50} nM of the CI-III, CI-V or the Sc-I siRNA oligonucleotides for 1 day. Two days after siRNA treatment, cells were exposed to the indicated concentrations of paclitaxel for 48 h, and cell viability was determined by an \textit{in vitro} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Each data point represents the mean of three independent experiments; bars denote SD; * indicate differences from control at \textit{p} < 0.01.
lular CLU protein forms produced by either a truncated CLU mRNA (5) or by altered posttranslational modification and intracellular trafficking of the CLU protein form transcribed from the full-length conventional transcript (53) are related to cell death. Similarly, it was shown that forced expression of intracellular CLU fragments initiates the formation of protein aggregates and cell death (54). These CLU protein forms, however, are seemingly strictly related to cell exposure to apoptosis-inducing agents and therefore should not be directly related to the main CLU function under physiological conditions.

In support to our suggestion of an intracellular cytoprotective CLU function, the observed reduced plating efficiency and growth of the CLU knock down cancer cells is most likely related to a decreased ability of cells to adhere to a substrate. CLU has been reportedly implicated in cell-substrate interactions. More specifically, it has been shown that in LLC-PK1 renal epithelial cells CLU promoted cell aggregation and cell adhesion in a specific and dose-dependent manner (55), whereas in vascular smooth muscle cells, exogenously added CLU promoted cell migration (56). The inhibition of cell adhesion observed in CLU knock down cells may be partially related to growth inhibition as indicated by the observed up-regulation of p21 to cellular sensitization to apoptosis, as well as to the observed decreased levels of bcl-2, the p53 stabilization, and the accumulation of Bax in U-2 OS cells. Considering that bcl-2 down-regulation occurs in both KO HS and U-2 OS cells, we suggest that it does not depend on p53 status and occurs upstream of p53 stabilization. Interestingly, down-regulation of both bcl-2 and bcl-xL in glioblastoma cell lines resulted in spontaneous cell death (57) and CLU levels are positively related to the antiapoptotic molecule bcl-xL levels in human melanoma cell lines (Hoeller et al., personal communication). Detachment of epithelial cells from the extracellular matrix results in a form of apoptosis often referred to as anoikis. Interestingly, the bcl-2 protein expression is higher in rapidly adhering keratinocytes and nearly absent in slowly adhering cells (58), whereas detachment from the extracellular matrix or disruption of the cytoskeleton results in a significant down-regulation of bcl-xL in nonmalignant rat and human intestinal epithelial cells (59). Moreover, loss of endogenous p53 activity in normal epithelial cells transfected with dominant-negative mutated p53 inhibited anoikis, thus demonstrating the involvement of p53-dependent processes in anoikis-mediated cell death (60). Our data in U-2 OS cells are in line with a novel recently identified proapoptotic function of p53 that does not require activation by genotoxic agents and appears to be constitutively suppressed by bcl-2. Silencing of bcl-2 in colorectal cancer cells by siRNA induced massive p53-dependent apoptosis that requires as essential apoptotic mediators bax and caspase-2 proteins (61).

In summary, considering that: (a) the CLU gene is differentially expressed during carcinogenesis and tumor progression, (b) treatment of cancer cells with chemotherapeutic drugs results in CLU gene induction, and (c) the primary function of the 60- and 75-kDa CLU protein forms is cytoprotective, we suggest that CLU is a target for therapeutic inhibition in cancer. The siRNA oligonucleotides used in this study are potent tools for modulating the CLU gene expression and they may ultimately develop into attractive antitumor therapeutics.

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REFERENCES


CELL SENSITIZATION BY CLU GENE EXPRESSION SILENCING


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Ioannis P. Trougakos, Alan So, Burkhard Jansen, et al.

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