Clusterin-Mediated Apoptosis Is Regulated by Adenomatous Polyposis Coli and Is p21 Dependent but p53 Independent

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ABSTRACT

Clusterin is a widely expressed glycoprotein whose function remains somewhat enigmatic. Also known as apolipoprotein J, clusterin was initially characterized as an apoptosis-associated transcript after it was identified as testosterone-repressed prostate message 2 (1) and sulfated glycoprotein 2 (2) that is overexpressed in the epithelial cells of the regressing rat ventral prostate (3). Increases in clusterin mRNA and protein levels have since been consistently detected in apoptotic heart, brain, lung, liver, kidney, pancreas, and retinal tissue both in vivo and in vitro (1, 4–11). Moreover, clusterin-knockout mice show reductions of cell death in hypoxia-ischemia–induced brain damage (12–14). Clusterin function, however, has also been reported to be antiapoptotic and has been found to be overexpressed in progressing breast carcinomas, where its expression correlated inversely with the apoptotic index. Similarly, clusterin-knockout mice show an increase in autoimmune myocardial damage, suggesting an antiapoptotic function. Recent studies have shed some light on the dichotomous functions of clusterin, suggesting pro- and antiapoptotic functions may be related to nuclear and secreted isoforms, respectively, and that the function of clusterin may be context dependent. Overexpression of the secreted form of clusterin in human cancer cells caused drug resistance and protection against certain cytotoxic agents that induce apoptosis (15). Additional data suggest that a secreted form of clusterin acts as a molecular chaperone, scavenging denatured proteins outside cells following specific stress-induced injury such as heat shock. Other data, show that overexpression of a specific nuclear form of clusterin acts as a prodeath signal (16). Furthermore, studies of human colon cancer suggest a conversion from the nuclear form of clusterin to the cytoplasmic form, which may promote tumor progression (17). Recently, a link between the accumulation of the nuclear form of clusterin and anoikis induction in prostate epithelial cells was shown (18).

To better elucidate the functional role of clusterin, we examined its expression and localization in a panel of normal and neoplastic human colon cancer tissues, as well as a panel of human colon cancer cell lines. We then evaluated the effect of APC expression and chemotherapy, both known to induce apoptosis, on clusterin expression and associated apoptotic events. The effect of clusterin transfection, or alternatively, antisense clusterin transfection, on basal and chemother-apy-induced apoptosis was investigated. Because both p21 and p53 have been linked to regulation of apoptotic events, clusterin expression and induction of apoptosis was evaluated in the context of their expression using targeted knockout cell line models.

INTRODUCTION

Clusterin is a widely expressed glycoprotein whose function remains somewhat enigmatic. Also known as apolipoprotein J, clusterin was initially characterized as an apoptosis-associated transcript after it was identified as testosterone-repressed prostate message 2 (1) and sulfated glycoprotein 2 (2) that is overexpressed in the epithelial cells of the regressing rat ventral prostate (3). Increases in clusterin mRNA and protein levels have since been consistently detected in apoptotic heart, brain, lung, liver, kidney, pancreas, and retinal tissue both in vivo and in vitro (1, 4–11). Moreover, clusterin-knockout mice show reductions of cell death in hypoxia-ischemia–induced brain damage (12–14). Clusterin function, however, has also been reported to be antiapoptotic and has been found to be overexpressed in progressing breast carcinomas, where its expression correlated inversely with the apoptotic index. Similarly, clusterin-knockout mice show an increase in autoimmune myocardial damage, suggesting an antiapoptotic function. Recent studies have shed some light on the dichotomous functions of clusterin, suggesting pro- and antiapoptotic functions may be related to nuclear and secreted isoforms, respectively, and that the function of clusterin may be context dependent. Overexpression of the secreted form of clusterin in human cancer cells caused drug resistance and protection against certain cytotoxic agents that induce apoptosis (15). Additional data suggest that a secreted form of clusterin acts as a molecular chaperone, scavenging denatured proteins outside cells following specific stress-induced injury such as heat shock. Other data, show that overexpression of a specific nuclear form of clusterin acts as a prodeath signal (16). Furthermore, studies of human colon cancer suggest a conversion from the nuclear form of clusterin to the cytoplasmic form, which may promote tumor progression (17). Recently, a link between the accumulation of the nuclear form of clusterin and anoikis induction in prostate epithelial cells was shown (18).

To better elucidate the functional role of clusterin, we examined its expression and localization in a panel of normal and neoplastic human colon cancer tissues, as well as a panel of human colon cancer cell lines. We then evaluated the effect of APC expression and chemotherapy, both known to induce apoptosis, on clusterin expression and associated apoptotic events. The effect of clusterin transfection, or alternatively, antisense clusterin transfection, on basal and chemother-apy-induced apoptosis was investigated. Because both p21 and p53 have been linked to regulation of apoptotic events, clusterin expression and induction of apoptosis was evaluated in the context of their expression using targeted knockout cell line models.

MATERIALS AND METHODS

Human Tissues. Snap-frozen human colon cancers (Dukes’ stage C) and their cognate normal mucosal controls were obtained from the Moffitt Cancer Center tissue bank in a de-identified fashion with an Institution Review Board-approved protocol. Tissues were microdissected for purity and then exposed to cellular lysis buffers and Trizol for RNA extraction.

Cell Lines. Four different human colon cancer cell lines were obtained from the American Type Culture Collection (HT29, KM12C, HCT116, and SW620), and two targeted knockout cell lines were a kind gift of Dr. B. Vogelstein (HCT116p21+/− and HCT116p53+/−). The HT29 cell line deficient of APC but engineered with a zinc-inducible wild-type APC vector (HT29APC) was also a gift of Dr. Bert Vogelstein. HT29, KM12C, HCT116, and SW620 cells were cultured in RPMI medium with 10% fetal bovine serum. HT29APC, HCT116p21+/−, HCT116p53+/−, and their parental cells were cultured in McCoy’s 5A medium with 10% fetal bovine serum. Cells were treated with Zn2++ (12 μmol/L) 4, 8, and 16 hours or with 5-fluorouracil (5-FU; 5 μmol/L) and anti-Fas antibody (50 ng/mL) or with irinotecan (6 μmol/L) 24, 48, and 72 hours to induce apoptosis.

Transient Transfection of Clusterin. The plasmids p4 empty vector and p4/SGP2 (containing human clusterin full-length cDNA) were obtained from Dr. Saverio Bettuzzi (Parma, Italy). Transient transfection of p4 empty vector (as a mock) or p4/SGP2 were performed using FuGENE 6 Transfection Reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s protocol. After transfection with mock or clusterin 24 to 48 hours, cells were either harvested or treated by apoptotic stimuli for 48 hours, then harvested to detect clusterin protein expression by Western blotting or to perform apoptosis assays. Each transfection experiment was done in triplicate. Data are presented as a mean ± SE.

Western Blot Analyses. Cells were harvested in protein lysis buffer [20 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 1 mmol/L DTT, 5 μmol/L trichostatin A, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF, and complete protease inhibitors (Roche Applied Science, Indianapolis, IN)], and Western blotting was performed as described previously (19). All blots were performed in...
triplicate. Representative examples are shown in the figures. Antibodies used
include clusterin (Alexis, San Diego, CA) and p53 and p21 (BD Biosciences,
San Jose, CA).

Antisense Clusterin Oligonucleotide Transfection. Pretreated ~1 × 10³
HT29, HCT11.6 and HT29APC cells in 100-mm culture dishes, respectively,
for overnight. They were transfected with antisense clusterin oligonucleotides
or mismatch clusterin oligonucleotides, respectively. Phosphorothioate oligo-
nucleotides used in this study were purchased from Integrated DNA Technol-
ogies, Inc. (Corvald, IA). The sequence of the antisense clusterin clusterin
oligonucleotide used corresponded to the human clusterin translation initiation
site (5'-CAGCAGAGCTTCTCACATG-3'). A 2-base clusterin mismatch oligonucleotide
(5'-CAGCAGAGGTATTATCAT-3') was used as control.

Transfections were performed using the LipofECTamine Plus reagent
(Invitrogen, Carlsbad, CA). Cells were treated with either 500 and 1000
mmol/L antisense or mismatch clusterin oligonucleotides after preincubation
for 20 minutes with 10 μg/mL lipofectin in serum-free OPTI-MEM (Life
Technologies, Inc., Carlsbad, CA). Four hours after the beginning of
the incubation, for HT29 and HCT116 cells, the medium containing oligonucle-
otides and lipofectin was replaced with standard culture medium plus 5-FU (5
μmol/L) and Fas (50 ng/mL) once daily for 3 days. For HT29APC cells, the
medium was replaced with standard culture medium plus Zn²⁺ (120 μmol/L)
for 16 hours. Cells were harvested to detect clusterin protein expression by
Western blotting or to perform apoptosis assays. Each transfection
experiment was done in triplicate and repeated at least three times. Data are presented as
a mean ± SE. cDNA Synthesis and Real-time PCR. To measure clusterin mRNA
expression, RNA was isolated from snap-frozen human colon cancers (Dukes’
stage C), and their cognate normal mucosal controls or colon cancer cells
treated with different reagents by using TRIzol reagent (Invitrogen) according
to the manufacturer’s protocol. A quantitative primer/probe set was designed
to evaluate and to quantify clusterin mRNA. Reverse transcription of RNA
was performed using 15 units of Omniscript reverse transcriptase (Qiagen,
Valencia, CA), 1× cDNA synthesis buffer, 40 units of RNase inhibitor (Life
Technologies, Inc.), 5 mmol/L DTT, 1 mmol/L deoxynucleoside triphosphate
mixture, 0.5 μg of Oligo(dT) primer, and 2 μg of RNA. Primers and probes for
real-time PCR were designed using Primer Express software (Applied Biosys-
tems, Inc., Foster City, CA). Each primer set consisted of standard PCR
primers (temperature 58° to 60°C) designed to span gene exons to exclude any
possible genomic DNA contamination. Detection and quantitation of each gene
was accomplished using an amplitcon-specific fluorescent oligonucleotide
probe (temperature 68°C to 70°C), with a 5′-reporter dye (carboxyfluorescein)
and a downstream 3′-quencher dye (6-carboxy-tetramethylrhodamine). The
primers used for detection of clusterin had the following sequences, 5′-
CTATCTGCGGGTCACCAC-3′ (forward primer), 5′-CACAGTGACACCG-
GAAGGAAC-3′ (reverse primer), and 5′-FAM/TGGCTTCCCACACTTCT-
GACCCG/ATAMRA-3′ (probe). During PCR, the 5′-3′ nuclease activity of
TaqDNA polymerase releases the reporter, whose fluorescent signal is then
detected by the GeneAmp 5700 Sequence Detector System (ABI, Foster City,
CA). The PCR reaction was performed in a 96-well optical reaction plate with
optical caps. The reaction mixture consisted of 0.2 μmol/L each primer, 0.1
μmol/L fluorescent probe, 5 units of AmpliTaq Gold (ABI), 200 μmol/L each
daTTP, dCTP, and dGTP, 400 μmol/L dUTP, 5.5 mmol/L MgCl₂, 8% glycerol,
1 unit of AmpErase uracil N-glycosylase and 1× TaqMan Buffer A.
Amplification profile was as follows: 50°C for 2 minutes (for uracil N-glycosylase
digest of contaminating PCR ampiclons), 95°C for 10 minutes, followed by 40
cycles of 95°C for 15 seconds, and 60°C for 1 minute, 2 μL of cDNA were
assayed per well. Standards used for quantitation were plasmid vector DNA
containing the primer-specific coding region for clusterin. Standard values
were 10⁸, 10⁷, 10⁶, 10⁵, 10⁴, and 10 DNA molecules per well. Two assays were
performed on each cDNA sample, clusterin, and 3-histone H3.3 as a house-
keeping gene to normalize mRNA values. Quantitative PCR was performed in
an ABI 5700 sequence detection system.

Apoptosis Assays. To determine apoptotic fractions, cells were harvested at
different time point after treatment by apoptotic stimuli. Cells were then
analyzed by using the Annexin V-phycocerythrin plus 7-amino-actinomycin D
(BD Biosciences) apoptosis detection kit according to the manufacturer’s
protocol. The DNA damage experiments were done in triplicate for each cell
line. Apoptotic fractions were determined by flow cytometry. Apoptosis
was confirmed by terminal deoxynucleotidyltransferase-mediated dUTP nick end
labeling (TUNEL) staining with the In Situ Cell Death Detection Kit (TMR
red; Roche Applied Science) according to the manufacturer’s protocol and
immunoblotting with human clusterin antibodies (Alexis).

Immunocytofluorescence and TUNEL Assay. Pretreated ~1 × 10³
cells/well were exposed to treatment conditions as planned (some cells were
were treated 48 hours by apoptotic stimuli, some cells were transiently transfected
with clusterin 24 hours, and then treated another 48 hours with apoptotic
stimuli). Cells were fixed with acetonethanol (1:1, Sigma, St. Louis, MO)
for 3 minutes, then were blocked with PBS + 10% normal goat serum for 20
minutes. Cells were incubated with clusterin antibody (1:200; Alexis) in PBS
+ 10% normal goat serum for 2 hours, then the slides were washed with PBS
+ 0.1% Triton X-100 three times and were incubated with FITC (1:100; Vector
Laboratories, Inc., Burlingame, CA) in PBS + 10% normal goat serum for 1
hour and then washed again with PBS + 0.1% Triton X-100. Some slides were
dried and covered with coverslips in Vectashield mounting media of antifade/4,
6-diamidino-2-phenylindole (1:1; Vector Laboratories, Inc.). Immunofluo-
rescense was observed with a Leitz Orthoplan 2 microscope, and images were
captured by a charge-coupled device camera with Smart Capture program
(Vysis, Downers Grove, IL). Some slides were double staining by TUNEL
assay with the In Situ Cell Death Detection Kit (TMR red; Roche Applied
Science) according to the manufacturer’s protocol to record clusterin expres-
sion and apoptotic cell death co-localization.

RESULTS

Clusterin Is Down-regulated in Human Colon Cancer. To further
explore the potential role of clusterin in colon cancer, the levels
of clusterin mRNA and protein were assessed in human colon neo-
plastic specimens (Dukes’ stage C) relative to matched normal mu-
cosal controls from 10 patients. Compared with the matched normal
samples, clusterin mRNA and protein expression were generally down-
regulated in human colon cancers (Fig. 1, A and B). Both RNA and
protein expression were consistently reduced by >50% in the major-
ity of cases.

Clusterin Is Up-regulated in Chemotherapy-induced Colon
Cancer Cell Apoptosis. To determine whether clusterin expression
was implicated with colon cancer cell apoptosis, we used two com-
mon chemotherapeutical agents for colon cancer, 5-FU (5 μmol/L)
and irinotecan (6 μmol/L) for proapoptotic stimuli. Anti-Fas antibody
(50 ng/mL) was added to 5-FU because we have previously shown

Fig. 1. Clusterin is down-regulated in human colon cancer. A, real-time quantitative
PCR assay of clusterin mRNA expression in human colon neoplastic specimens (Dukes’
stage C) relative to matched normal mucosal controls from 10 patients. B, Western blot
analyses for clusterin protein expression in the same patient samples. Compared with the
matched normal tissues, clusterin mRNA and protein expression were generally down-
regulated in human colon cancers. Real-time PCR data were normalized by mRNA level
to glyceraldehyde-3-phosphate dehydrogenase.

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that the combination enhances apoptotic responses in colon cancer cell lines. After treatment, HT29, HCT116, KM12C, but not SW620 had significant increases in apoptosis (Fig. 2A). Apoptosis was assessed by flow cytometric analysis using Annexin V-phycoerythrin. Quantitative PCR and Western blotting were then used to measure clusterin mRNA and protein expression. Clusterin protein expression at baseline was undetectable for all four cell lines and dramatically increased with chemotherapy exposure in all cell lines but SW620 (Fig. 2B). Fluorescence immunostaining and TUNEL staining were used to localize clusterin expression and apoptotic cell death. Clusterin was found to be primarily expressed in the nuclei in the TUNEL-positive stained apoptotic cells after chemotherapy treatment. The results indicated that the fraction of apoptotic cells was increased ~5-fold in those cells exposed to apoptotic stimuli relative to controls absent of apoptotic stimuli in HT29 (Fig. 2C) and HCT116 (Fig. 2D), but again, no apoptosis was induced at SW620 colon cancer cell line (data not shown).

Clusterin Is Up-regulated in Wild-type APC-Induced Colon Cancer Apoptosis. Because most colon cancers lack functional APC protein and the data suggest diminished clusterin expression in these samples, we turned to a well-described in vitro cell line model in which wild-type APC protein can be induced to cause apoptosis. A null APC−/− human colon cancer cell line (HT-29) stably transfected with a Zn2+-inducible wild-type APC vector or a β-galactosidase control, was used to additionally investigate the relationship between clusterin expression and APC. As demonstrated by real-time quantitative PCR, the clusterin mRNA levels dramatically increased in cells as early as 4 hours after induction of wild-type APC expression. (B) Similarly, protein levels of clusterin were shown to be up-regulated with induction of wild-type APC expression early after APC induction.

Fig. 2. Clusterin is up-regulated in chemotherapy-induced colon cancer cell apoptosis. A. To determine whether clusterin expression was implicated with colon cancer cell apoptosis, we used 5-FU (5 μmol/L) plus anti-Fas antibody (50 ng/mL) as proapoptotic stimuli. After treatment for 48 hours, apoptotic cell death was measured by an FACS flow cytometer using Annexin V-phycoerythrin plus 7-amino-actinomycin D and analyzed by ModFitLT software. Each value represents the mean ± SE (n = 3). *Student t test, P < 0.01 versus untreated control. HT29, HCT116, KM12C, but not SW620 had significant increases in apoptosis with treatment. B, real-time quantitative PCR results showed that clusterin mRNA was up-regulated for all cell lines except SW620, compared with untreated controls. A Western blot analysis showed that clusterin protein expression at baseline was undetectable for all four cell lines and dramatically increased with chemotherapy exposure in all cell lines but SW620. C and D, dual-staining immunofluorescence assay and TUNEL staining stained clusterin antigen (green), apoptotic cell death (red), and nuclei (blue). Clusterin was found to be primarily expressed in the nuclei in the TUNEL-positive stained apoptotic cells after chemotherapy treatment in HT29 and HCT116. DAPI, 4',6-diamidino-2-phenylindole.
induction of wild-type APC expression (Fig. 3A). Similarly, protein levels of clusterin were shown to be up-regulated with induction of wild-type APC expression early after APC induction (Fig. 3B). This induction of clusterin and APC tightly correlate with the induction of apoptosis in these cells (data not shown).

Antisense Clusterin Oligonucleotide Reduced Chemotherapy- or APC-Induced Apoptosis. To understand if clusterin expression directly linked to chemotherapy- or APC-induced apoptosis, we used previously described (20) antisense clusterin oligonucleotides to test whether blocking clusterin expression by antisense clusterin oligonucleotide could reduce apoptosis induced by chemotherapy or APC. The results showed that clusterin antisense clusterin oligonucleotides (1 mmol/L), but not mismatch control oligonucleotides, significantly decreased clusterin mRNA and protein expression induced by pro-apoptotic stimuli (Fig. 4A and B). Similarly, apoptotic cell death induced by pro-apoptotic stimuli was decreased by ASO >50%, compared with MM control oligonucleotides in HT29, HCT116, and HT29 APC cells.

Fig. 4. Antisense clusterin oligonucleotide (ASO) reduced chemotherapy- or APC-induced apoptosis. A and B. Real-time PCR data showed that clusterin ASO (1 mmol/L), but not mismatch (MM) control oligonucleotides (1 mmol/L), significantly decreased clusterin mRNA (P < 0.01 ASO versus MM control oligonucleotides), and protein expression induced by pro-apoptotic stimuli. C. Similarly, apoptotic cell death induced by pro-apoptotic stimuli was decreased by ASO >50%, compared with MM control oligonucleotides in HT29, HCT116, and HT29 APC cells.

Experimental Groups

Transient Transfection of Clusterin Localized to the Nucleus and Directly Induced Apoptosis or Enhanced Chemotherapy-Induced Apoptosis in Colon Cancer Cells. Given the correlation between clusterin overexpression and cell death, we investigated whether clusterin directly contributed to colon cancer cell death. We
transiently transfected clusterin to the wild-type HT29 cell line. After 24 or 48 hours, clusterin protein expression increased ~10-fold compared with mock-transfected or wild-type cells (Fig. 5A). We used fluorescence immunostaining and TUNEL staining to detect clusterin expression and cell apoptosis principally in nucleus of the transfected cells. Clusterin antigen (green), apoptosis cell death (red), and nuclei (blue). C. Flow cytometric analysis showed that transient transfection of clusterin induced apoptosis in >30% cells, as compared with the mock-transfected or wild-type cells, and (D) transiently transfected clusterin enhanced apoptosis by ~2-fold in cells treated with 5-FU and Fas as compared with mock-transfected cells treated with 5-FU and Fas (Fig. 5D). These results indicated that transient transfection of clusterin to colon cancer cells either directly induced apoptosis or enhanced chemotherapy sensitivity in human colon cancer cells.

Clusterin Induction in Chemotherapy or APC-Induced Colon Cancer Cell Apoptosis Was p21 Dependent. As a broad-acting cyclin-dependent kinase inhibitor, p21 (WAF1) occupies a central position in the cell cycle regulation of self-renewing tissues. In addition to regulating normal cell cycle progression decisions, p21 integrates genotoxic insults into growth arrest and apoptotic signaling pathways that ultimately determine epithelial cell fate. We have found that chemotherapeutical agents or APC were able to substantially up-regulate p21 expression (Fig. 6A). Because we knew that these treatments also induced clusterin and apoptosis, we investigated clusterin expression in wild-type HCT116p21+/− and knockout HCT116 p21−/− cells. When HCT116 p21−/− cells were treated with proapoptotic stimuli (5-FU or irinotecan), both clusterin and p21 expression were detected. Absent of p21 expression, however, clusterin was not expressed. Similarly, HCT116p21−/− cells showed significant resistance to 5-FU and Fas-induced apoptosis as compared with the parental cells (HCT116p21+/−; Fig. 6B). These results demonstrated that without p21 expression, clusterin was not induced and apoptosis was inhibited. When clusterin was transiently transfected into HCT116 p21−/− cells, however, clusterin protein was overexpressed and colocalized in the nucleus with apoptotic cells. The percentage of apoptotic cells increased ~6-fold as compared with control cells. Clusterin expression effectively bypassed the requirement for p21 expression, inducing basal levels of apoptosis, and enhancing chemotherapy-induced apoptosis (Fig. 6C). Using the colon cancer cell line SW620, we previously showed its resistance to chemotherapy-induced apoptosis (Fig. 2A) and its absence of clusterin expression (Fig. 2B). Here, we show that the SW620 cell line has no detectable p21 expression nor clusterin expression either with or without chemotherapy treatment (Fig. 6D), potentially explaining why this cell line is resistant to chemotherapy induced apoptosis. Taken together, our results suggest that clusterin acts downstream of p21 and that clusterin overexpression in chemotherapy-induced apoptosis is p21 dependent.

Clusterin and p21 Induction in Chemotherapy-Induced Colon Cancer Cell Apoptosis Are p53 Independent. The tumor suppressor gene p53 is the primary transcription factor for the induction of p21. To determine whether p53 expression is implicated to p21-clusterin signal transduction pathway, we evaluated p53 expression in HT29 (p53 mutant) and HCT116 (p53 wild-type) colon cancer cell lines. We found that p21 and clusterin were overexpressed, but p53 levels were unaffected in cells after chemotherapeutic treatment (Fig. 7A). We also detected p21 and clusterin expression in HCT116 p53−/− cells after treatment with chemotherapy (Fig. 7B). The results suggest that clusterin and p21 up-regulation by chemotherapeutic treatment is independent of p53 expression.

DISCUSSION

Although clusterin was originally proposed to be a marker for programmed cell death, it has variously been proposed to provide both pro- and antiapoptotic functions. Studies of clusterin-transfected renal carcinoma cells suggest that clusterin expression may increase cell survival, motility, invasive capacity, and contribute to metastasis (14). In ApcMin mice, both colon neoplasia, tumor cells undergoing apoptosis expressed low levels of clusterin (21). In cancer patients, overexpression of clusterin has been linked to tumor progression in both breast and colon cancer (17, 22). These data support an antiapoptotic role for clusterin. Conversely, a number of studies also support a
proapoptotic role for this molecule. Previous studies suggest that clusterin expression decreases the proliferation rate of prostate epithelial cells (23) and is reduced in human prostate cancer (24), consistent with a proapoptotic function. In addition, knockout studies show reductions in cell death under stress conditions (14, 25).

Our investigations sought to clarify the functional role of clusterin in human colon cancer. Only recently has it come to light that two forms of clusterin, a secreted and a nuclear form, may be responsible for this divergent behavior. It is now apparent that the endogenous nuclear clusterin is a product of alternative splicing. The shorter

![Flow Cytometry](image)

Fig. 6. Clusterin induction in chemotherapy- or APC-induced colon cancer cell apoptosis was p21 dependent. A. Western blot analysis showed that chemotherapeutic agents or APC were able to substantially up-regulate p21 protein expression. B. Western blot analysis showed that when HCT116 p21−/− cells were treated with proapoptotic stimuli (5-FU or irinotecan), both clusterin and p21 expression were detected. Absent of p21 expression, however, clusterin was not expressed in HCT116 p21−/− cells. Flow cytometric data showed that HCT116p21−/− cells showed significant resistance to 5-FU and Fas-induced apoptosis, as compared with the parental cells (HCT116p21+/+). C. Western blot analysis showed that with transient transfection of clusterin into HCT116 p21−/− cells, clusterin protein was overexpressed. Fluorescence immunostaining and TUNEL staining showed that the overexpression of clusterin was colocalized in the nucleus with apoptotic cells. Flow cytometric data showed that the percentage of apoptotic cells increased ~6-fold as compared with control cells. D. Western blot analysis showed that the SW620 cell line had no detectable p21 expression nor clusterin expression either with or without chemotherapy treatment.
mRNA coding for nuclear clusterin produces a Mr 49,000 precursor protein that acts as a prodeath signal, inhibiting cell survival and cell growth. It contains a nuclear localization signal with potential for targeting it to the nucleus where it can become activated as a mature Mr ~55,000 death protein under conditions of significant stress (16).

Previous studies of colon cancer found an overexpression of secretory clusterin in the cytoplasm but a concurrent decrease in nuclear clusterin (17). Although our data show a decrease in clusterin in cancers relative to normal mucosal controls, they are consistent with these results in that we identified a significant decrease in clusterin expression, using antibodies recognizing the nuclear isoform of clusterin, when comparing Dukes’ stage C colon cancers to their matched normal tissues. This decrease in expression linked to tumor progression is also consistent with the role of clusterin as a tumor suppressor gene, normally providing proapoptotic function. Taken together, these results suggest that the discrepancies in previous studies of human cancer clusterin expression are likely related to the type of clusterin measured.

We sought to additionally investigate the relationship between clusterin expression and apoptotic function by examining clusterin expression in the context of APC expression and chemotherapy exposure. Because APC is mutated in the vast majority (>95%) of sporadic colon cancers and APC expression has been linked to the induction of apoptotic events (19), we investigated the role of clusterin in APC-induced apoptosis. We identified that apoptosis induced by APC in APC-null colon cancer cells or by chemotherapy was linked to significant increases in clusterin expression. These data suggested that it was the nuclear form of clusterin (proapoptotic form) that contributes to human colon cancer apoptosis by colocalizing clusterin to apoptotic nuclei. In addition, we found that the stress induced by chemotherapies readily elevated clusterin levels and produced apoptosis.

Because clusterin has been proposed to regulate the cell cycle, increasing the accumulation of cells in the G_0-G_1 phases of the cell cycle (14), we elected to evaluate the potential relationship between p21 and p53 and clusterin expression. The finding that targeted inactivation of p21 enhances Apc-initiated tumor formation (26) also led us to study this relationship further. Our data suggest that clusterin is induced in cells undergoing apoptosis stimulated by APC expression or by chemotherapy exposure. Moreover, we found a coordinate up-regulation of p21 and clusterin expression in cells undergoing apoptosis in response to these stimuli. The question then arose as to whether p21 expression was necessary for clusterin expression. To examine this question, we evaluated human colon cancer cells with a targeted deletion of p21 (HCT116p21^-/-) and found that when treated with proapoptotic stimuli, no clusterin overexpression was seen and significantly less apoptosis was induced. Interestingly, the only cell line that was not susceptible to chemotherapy-induced apoptosis, SW620, was also incapable of mounting a p21 response, presumably preventing clusterin expression. Furthermore, when clusterin was transiently transfected into HCT116 p21^-/- cells, clusterin protein was overexpressed in the nucleus and colocalized with TUNEL staining in the apoptotic cells. The percentage of apoptotic cells increased ~6-fold as compared with control cells, suggesting that clusterin can effectively bypass the action of p21 and induce apoptosis in the absence of p21. We also found that p21 and clusterin were overexpressed, but p53 levels were unaffected in cells after chemotherapeutic treatment. Similarly, p21 and clusterin were overexpressed in HCT116 p53^-/- cells after stimulation by chemotherapy (Fig. 6, I and J). Taken together, these results suggest that clusterin acts downstream of p21 and that clusterin overexpression in chemotherapy-induced apoptosis was p21 dependent and p53 independent. This work is the first to demonstrate a novel link between the nuclear form of clusterin overexpression and the induction of p21.

The p21 signaling pathway that regulates the nuclear form of clusterin expression after chemotherapy exposure is unknown. Induction of the cyclin-dependent kinase inhibitor p21 is a common mechanism of growth arrest in different physiologic situations. Ectopic overexpression of p21 leads to cell growth arrest in G1 and G2, possibly by inhibition of cyclinB1/cyclin-dependent kinase 1 as demonstrated in prostate cancer cells. This arrest is accompanied by apoptotic cell death. Although p21 is not a transcription factor, it is conceivable that indirect effects of p21 on cellular gene expression may mediate some of its functions. For example, transient transfection assays showed that p21 could stimulate nuclear factor-κB–mediated transcription; this effect of p21 has been explained through the interaction of cyclin-dependent kinase 2 with transcriptional cofactors from 300 that augments nuclear factor-κB and other inducible transcription factors. p21 interactions with proteins other than CDK may also have a potential effect on gene expression. For example, p21 was reported to bind c-Jun NH2-terminal kinases, apoptosis signal-regulating kinase 1, and Gadd45 (27). It was already shown that clusterin promoter region has AP-1 protein (c-Jun and c-Fos) binding sites. In a study by Jin et al. (28), transforming growth factor-β induced clusterin expression in a variety of cell types via a consensus AP-1 binding site.

Collectively, the presented data demonstrate that overexpression of the nuclear form of clusterin either enhanced chemotherapeutic sensitivity or directly induced colon cancer apoptosis. The induction of clusterin by chemotheraphy was p21 dependent but p53 independent. Because clusterin transfection reverted the absence of p21 and caused apoptosis, clusterin protein appears to be an important protein for regulating chemotherapeutic-induced apoptosis and may have a potential therapeutic role.

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