Ethylenediaminetetraacetic acid affects subcellular expression of clusterin protein in human colon adenocarcinoma COLO 205 cell line

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The aim of our study was to determine the expression of various isoforms of clusterin and to evaluate how etoposide or calcium chelators [ethylenediaminetetraacetic acid and (2-aminoethoxyethane)-*N,N,N′,N′*-tetraacetic acid] affect the subcellular expressions of the 50-kDa isoform of clusterin protein in colon adenocarcinoma COLO 205 cells. We then determined how the cytoplasmic vs. nuclear expression of the 50-kDa isoform of clusterin correlates with the viability of COLO 205 cells. To identify the clusterin isoforms, and its nuclear and cytoplasmic expression in COLO 205 cells, Western bloting was used. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide assay. Etoposide decreased the viability of COLO 205 cells with a concomitant increase in the 50-kDa clusterin concentration in the cell nucleus. Chelation of the extracellular calcium ions by (2-aminoethoxyethane)-*N,N,N′,N′*-tetraacetic acid did not modulate the subcellular distribution of clusterin. The use of ethylenediaminetetraacetic acid, which reduces the intracellular and extracellular calcium levels, stimulated nuclear expression of clusterin protein and was accompanied by extensive cell death. Intracellular calcium determines cytoplasmic expression and antiapoptotic activity of the intracellular protein clusterin. The depletion of intracellular calcium leads to increased nuclear expression of the 50-kDa clusterin protein, which is accompanied by cell death. We concluded that there is at least one cell death-promoting pathway in COLO 205 cells that is dependent on intracellular calcium and nuclear localization of 50-kDa clusterin.

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Introduction

It is widely known that owing to the expression of several antiapoptotic proteins, colorectal cancers are immune to death by natural ligands through the extrinsic apoptotic pathway. The resistance of colorectal cancers to death stimuli and the role of particular anti-apoptotic or proapoptotic proteins, however, still remain unclear. Among the proteins that are involved in the regulation of apoptosis, the protein clusterin (CLU) seems to be of considerable importance [1–4]. According to Caccamo *et al.* [5], CLU protein seems a logical candidate to become the missing link between the cell death machinery and calcium homeostasis. The protein CLU is also known as apolipoprotein J, testosterone-repressed prostate message-2 or sulfated glycoprotein-2. CLU is involved in several physiological processes that are important for tumor development (for details see the review article by Pajak and Orzechowski [6]). Cell cycle regulation, DNA repair, cell adhesion, resistance to chemotherapy, tissue remodeling, lipid transformation, membrane recycling, immune system regulation and apoptotic cell death are all considered targets for CLU activity [7–9]. Interestingly, two distinct isoforms of CLU that possess dual activity were identified. If the translation starts from the first AUG codon in CLU mRNA, it results in the synthesis of 60-kDa CLU. This precursor form is then transported to the endoplasmic reticulum by the leader peptide and glycosylated. It is then cleaved into α- and β-subunits bound together by five disulfide bonds [10]. Regardless of species, the mature, highly conserved, 80-kDa isoform of CLU could be secreted in extracellular space and scattered in body fluids. This isoform acts as a molecular chaperone following stress-induced injury such as heat shock and scavenges denaturated proteins outside the cells [11]. This secreted and cleaved isoform (sCLU, 40 kDa) also acts as a membrane scavenger, and has been implicated in causing drug resistance and protection against certain cytotoxic agents that normally induce apoptosis [12,13]. The second translation starts from the second AUG codon in the CLU mRNA sequence and leads to the synthesis of the 50-kDa intracellular isoform of CLU (iCLU), which is involved in apoptosis. Several published studies have demonstrated that the retention of 50-kDa

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iCLU in the cytoplasm correlates positively with cell survival, whereas its translocation to the nucleus promotes cell death [14,15]. More importantly, CLU upregulation has been observed and confirmed in several human malignancies, including bladder [16], prostate [5,14,15,17], breast [18,19] and colon [13,20] cancers. In-vitro and in-vivo studies performed on colorectal cancers demonstrated a decreased level of nuclear CLU, which was related directly to the higher viability, aggressiveness and enhanced metastatic potential of the cancer cells. Additionally, this unfavorable *status quo* was supported by the overexpression of secretory CLU or localized in the cytoplasm, iCLU. Chen *et al.* [1] have shown elevated expression of CLU in the tumor-bearing gut, when compared with normal tissue. Furthermore, the immunohistochemical staining showed the correlation between CLU expression and redistribution with tumor stage and grade [13]. Moreover, Pucci *et al.* [13] performed in-vitro experiments on the Caco-2 cell line to determine whether the expression of the nuclear proapoptotic CLU isoform is involved in apoptosis induction in colon cancer cells. Western blot analyses revealed that incubation of the cells with somatostatin caused a marked decrease in the 50-kDa CLU in the cytoplasm with a concomitant increase in this isoform within the nucleus. The latter was positively correlated with cell death. Similar experiments performed on freshly isolated colon cells isolated from a human low-grade carcinoma confirmed previous results. Moreover, the glycosylation assay revealed nuclear localization of nonglycosylated CLU isoform after somatostatin treatment. Analogous results have been shown by Chen *et al.* [1]. After treatment with chemotherapeutic compounds, apoptosis was induced in HT 29, HCT 116 and KM12C colon cancer cells. The number of apoptotic cells was directly related to the increased level of nonglycosylated CLU (iCLU within the nucleus).

Nuclear translocation of iCLU could be induced by various stimuli, such as ionizing radiation [17], chemotherapeutical drugs (etoposide) [15,21] or cytotoxic cytokines. According to Caccamo *et al.* [5], the depletion of calcium ions in PNT1A prostate cancer cells also modulates cellular retention and apoptotic activity of CLU. The first suggestions indicating proapoptotic activity of CLU were reported by Leskov *et al.* [10], O'Sulivan *et al.* [19], Caccamo *et al.* [21] and Criswell *et al.* [22]. In colon cancer cells resistant to apoptosis, the intracellular CLU was found mainly in the cytoplasm, whereas in normal cells the nuclear staining of CLU was revealed [13]. Similar results were obtained from prostate PC3 cancer cells [15]. In PNT1A [21] and PC3 [15] prostate cancer cells, the apoptotic activity of 50-kDa CLU was induced by etoposide treatment (50 and 100 μmol/l). Scaltriti *et al.* [15] observed that the etoposide-induced apoptosis is mediated by caspase-3 activation, because its proapoptotic activity was reduced after administration of pan-caspase inhibitor – benzyl-oxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk). Hitherto, there appears to be little or no data indicating the role of calcium in proapoptotic activity of CLU in colon adenocarcinoma cell line COLO 205.

Therefore, in these studies, the human colon adenocarcinoma COLO 205 cell line, naturally resistant to TRAIL-induced [23,24] and tumor necrosis factor-α-induced apoptosis [25,26], was used. Our previous studies from COLO 205 cells have shown the presence of antiapoptotic proteins, such as FLIP, that by interaction with tumor necrosis factor receptor 1 signalosome blocked the transduction of death signal [25]. We also found that modulation of calcium homeostasis could influence survival of COLO 205 cells.

With these data in mind, in the present study, we hypothesized that CLU expression and localization, and its effect on apoptotic activity, could be regulated by calcium ions.

Methods

Reagents

All reagents, dimethyl sulfoxide (DMSO), Tris, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, ethylenediaminetetraacetic acid (EDTA), (2-aminoethoxyethane)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), sodium chloride (NaCl), bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), dithiothreitol, etoposide and 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT), were cell culture tested, of high purity and, unless otherwise stated, purchased from Sigma-Aldrich (St Louis, Missouri, USA). Reagents for experimental applications were prepared according to the manufacturer's recommendations and, if possible, stored as stock solutions (1000-fold the highest working concentration). Primary polyclonal rabbit anti-CLU IgG antibody and secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Other reagents were purchased as stated in the description of the respective methods (see the text). Sodium dodecyl sulfate 100 g/l, sequi-blot polyvinylidene fluoride membrane 0.2 μm and all reagents for immunoblotting were obtained from Bio-Rad (Hercules, California, USA). Sera, media and antibiotics were obtained from Gibco Life Technologies (Paisley, UK).

Cell culture

Human colon adenocarcinoma cell line COLO 205 was purchased from American Type Culture Collection (ATCC Manassas, Virginnia, USA). Cells were maintained in the exponential phase of growth in growth medium [100 ml/l fetal bovine serum/Dulbecco's modified Eagle's medium (DMEM) with Glutamax and antibiotic–antimycotic mixture (Penicillin G sodium salt 50 IU/ml,

streptomycin sulfate 50 μg/ml, gentamycin sulfate 20 μg/ml, fungizone-amphotericin B 1 μg/ml)]. The cells were grown at 37°C, in a controlled, humidified 50 ml/l CO_2 atmosphere, on a 96-well flat-bottomed or tissue culture Petri dishes (100-mm diameter; BD Biosciences, Franklin Lakes, New Jersey, USA).

Experimental procedure

During propagation, medium was changed every other day until cultures reached 100% confluence. One day (24 h) before the experiment, confluent cells (cells of the same cell density fully covering the surface of the dish) were then switched to post-mitotic status to induce quiescence (withdrawal from cell cycle) by replacing growth medium with 20 g/l BSA/DMEM designated control medium. In the above-mentioned conditions, divisions of COLO 205 cells have been completed. During the study, freshly prepared media with or without experimental factors were changed according to the experimental schedule.

Cell viability

Assessment of cell viability on the basis of mitochondrial function was assayed by the ability of cells to convert soluble MTT into an insoluble purple formazan reaction product with minor modifications to the protocol described by Jacobson *et al.* [27]. For this assay, during the last 1 h of incubation time, the media were replaced by MTT solution (5 mg/ml in DMEM without phenol red, sterilized by filtration). MTT solution was then aspirated and formazan in cells was instantly dissolved by addition of 100 μl DMSO. In the case of EDTA, 100 μl of supernate was transferred to separate 96-well multiplate and 200 μl DMSO was added to dissolve formazan by repetitive pipetting. In all cases, cells were examined under phase-contrast microscopy before application of MTT to visually assess the degree of cell death. The absorbance was measured at 570/630 nm with an enzyme-linked immunosorbent assay reader, type Infinite 200 (TECAN, Grodig, Salzburg, Austria). Percentage cell viability [MTT conversion into purple formazan in comparison with Ctrl (20 g/l BSA/DMEM) or 1 ml/l DMSO in control medium] indicates cell viability (mitochondrial respiration or activity of mitochondrial dehydrogenases).

Apoptotic index and detection of apoptotic cells using morphological criteria

Cells were grown on the Lab-Tek 4-Chamber Slide w/Cover (Permanox Slide Sterile; Nalge Nunc International, Naperville, Illinois, USA). Cytotoxicity with resultant cell death was evaluated by microscopic observations. Apoptosis was evaluated by in-situ uptake of propidium iodide (PI) and bisbenzimide (Hoechst 33342), as described by McKeague *et al.* [28]. The cells were then washed very gently (to avoid detachment) with medium (the more dead cells, the fewer the cells that remained attached), and the media from a particular treatment with detached cells and cell debris were collected and centrifuged (12 000 *g*, 15 min). Next, attached or detached cells were stained by both PI followed by Hoechst 33342 staining to distinguish live, necrotic, early-apoptotic and late-apoptotic cells. Afterwards, the cells were fixed with an equal volume of methanol/acetic acid (3 : 1 v/v); the cells were gently washed with ice-cold phosphate-buffered saline (PBS including Ca^{2+} and Mg^{2+}) and mounted on slides using mounting medium prepared according to the manufacturer's protocol (Mowiol; CN Biosciences, La Jolla, California, USA). A BX-60 Olympus fluorescent microscope (Olympus, Melville, New York, USA) equipped with a PM20 automatic photomicrograph system, was used for photographic recording. At least 100 nuclei were counted under ultraviolet irradiation in 10 (or more if necessary) randomly chosen visual fields per slide. In the same visual field, the excitation of PI should lead to the appearance of red nuclei of the dead cells (no such cells have been detected). Cells were considered apoptotic if they were PI-negative and chromatin was condensed at the periphery of the nuclei. Apoptotic index (AI) was calculated from the number of apoptotic nuclei vs. total number of nuclei at each visual field ($n = 10$). Three independent experiments were performed.

Sample preparation for electrophoresis and immunoblotting

Cells were grown on 100-mm diameter culture Petri dishes. Following each experiment, the cells were washed twice with PBS, scraped off in PBS and centrifuged (4°C, 10 000 *g*, 5 min). Cell pellets were stored at −80°C to the end of the experiment. Cell pellets were resuspended in 400 μl of ice-cold buffer (10 mmol/l 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid pH 7.9; 10 mmol/l KCl; 0.1 mmol/l EDTA; 0.1 mmol/l EGTA; 1 mmol/l dithiothreitol; 0.5 mmol/l PMSF) and were then incubated on ice for 15 min, after which 25 μl of a 100 ml/l solution of Igepal CA-630 was added. After centrifugation (room temperature, 1000 *g*, 30 s), supernatants containing the cytoplasm were transferred to fresh tubes and were stored at −20°C. Nuclear pellets were resuspended in 200 μl RIPA buffer [1 × PBS; 10 ml/l Igepal CA-630; 5 g/l sodium deoxycholate; 1 g/l sodium dodecyl sulfate; aprotinin solution (Sigma-Aldrich) 30 μl added to 1 ml of buffer; 1 mmol/l sodium orthovanadate] and were passed through a 21-gauge needle. PMSF (0.1 mg/ml) was added and cells were incubated for 30 min on ice. After centrifugation (4°C, 10 000 *g*, 5 min), nuclear lysates were stored at −80°C until analysis. Soluble protein concentrations in the cytoplasmic and in the nuclear fraction were determined by a protein–dye binding method [29] with a commercial reagent (Bio-Rad).

Electrophoresis and immunoblotting

Equal amounts of sample protein (either 50 or 30 μg) isolated from the treated or untreated COLO 205 cells were

then resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting. Electrotransfer of proteins to polyvinylidene fluoride membranes (0.2 μm) was performed for 1.5 h at 100 V and followed by overnight blocking (4°C) in TBS buffer (20 mmol/l Tris, 500 mmol/l NaCl, pH 7.5), supplemented with 50 g/l non-fat powdered milk. Membranes were stained with Poinceau S dye to check equal and homogeneous loading. After washing in TBST (TBS containing 0.5 ml/l Tween 20), the membranes were immunostained by standard methods provided by the manufacturer; Santa Cruz Biotechnology). They were probed with a primary antibody (rabbit anti-CLU polyclonal antiserum; Santa Cruz Biotechnology) for 1 h at 20°C or overnight at 4°C, washed three times in TBST and were further incubated with the secondary donkey anti-rabbit antibody conjugated with horseradish peroxidase (see Reagents). Membranes were also probed with goat polyclonal anti-β-actin antibody to normalize proteins level. The blots were developed using the enhanced chemiluminescence detection system (Amersham International, Aylesbury, UK) according to the manufacturer's protocol. After exposure, photographs were taken with a Kodak DC 290 zoom digital camera, (Eastman Kodak Company, Rochester, New York, USA) and were scanned and analyzed using the Kodak EDAS 290/Kodak 1D 3.5 system.

Statistical analysis

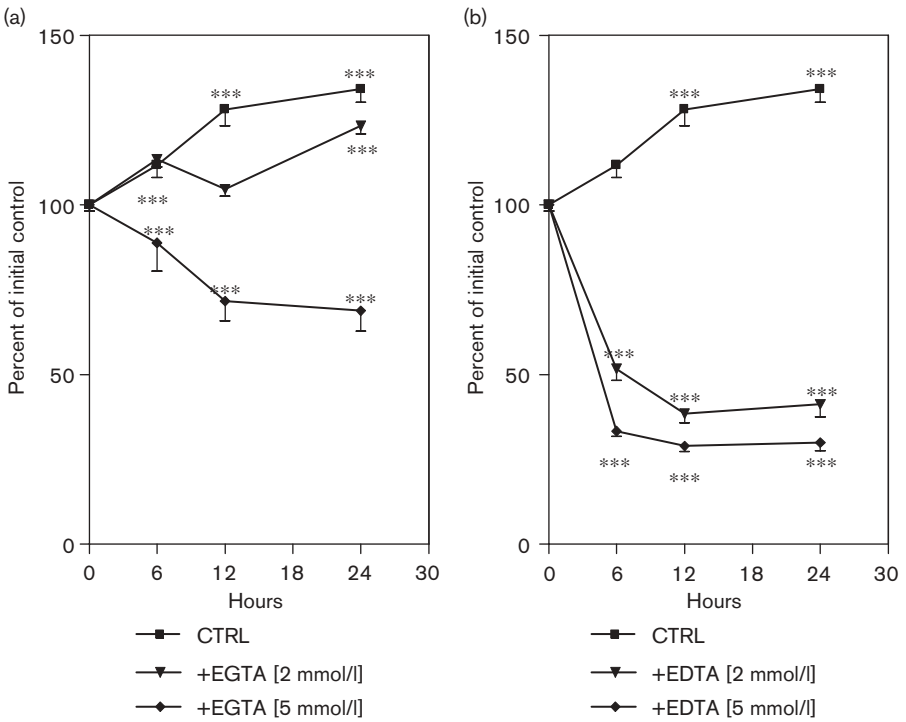
Each treatment was carried out in triplicate and each experiment was repeated at least twice. The results were statistically evaluated with one-way analysis of variance and Tukey's multiple range test, when compared with control treatments, or by two-way analysis of variance with Benferroni post-test to compare replicate means between the treatments. These analyses were performed using GraphPad Prism version 4.03 software (GraphPad Software, San Diego, California, USA). In order to show the quantitative differences, percentage of initial control values set arbitrarily as 100% (experimental value/initial control value × 100) at each time point were used. Statistical differences were interpreted as significant at $P < 0.05$ and highly significant at $P < 0.01$.

Results and discussion

Calcium chelators modulate viability of COLO 205 cells

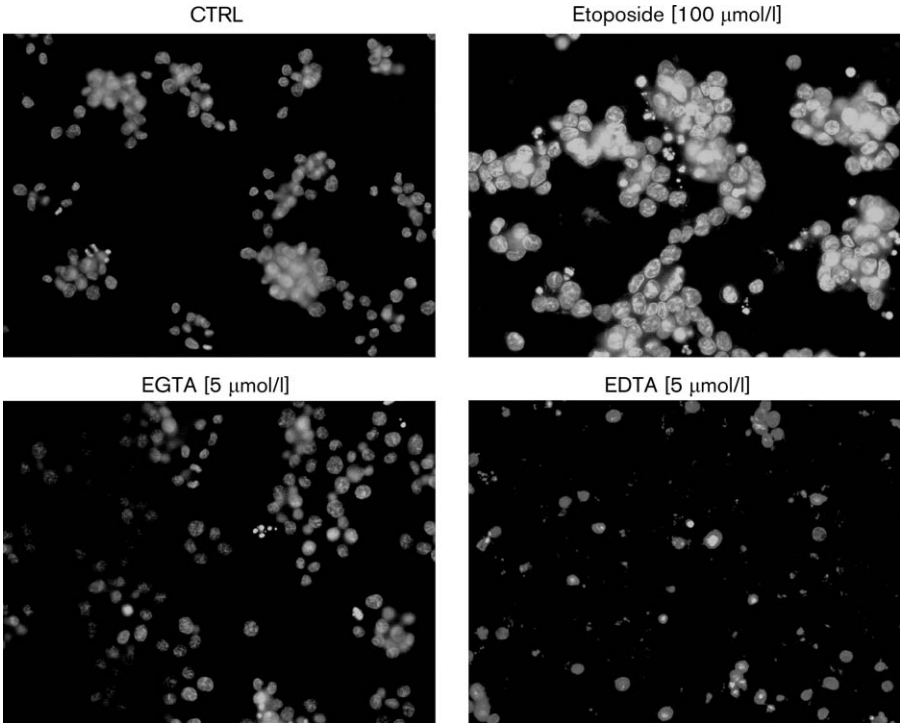
To investigate the influence of calcium ions on COLO 205 cell survival, two chelators were used: EGTA, an extracellular calcium chelator, and EDTA, which chelates both intracellular and extracellular calcium ions. As previously mentioned, the cells were cultured in control medium for 24 h, followed by treatment with 2 or 5 mmol/l EGTA or 2 or 5 mmol/l EDTA. After 6, 12 and 24 h, cell viability was measured by MTT assay. All concentrations of

Fig. 1



Dose–response curves of viability of COLO 205 cells in the presence of (a) (2-aminoethoxyethane)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (2 or 5 mmol/l) and (b) ethylenediaminetetraacetic acid (EDTA) (2 or 5 mmol/l). Viability was calculated as a percentage of initial control (CTRL) value at '0' time as determined by 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide staining. The studies were performed in quadruplicates and repeated at least twice with similar results. Significant differences between the means and control value are indicated by $*P<0.05$, $**P<0.01$ or $***P< 0.001$.

Fig. 2



Series of images obtained from a fluorescent microscope under ultraviolet light. Nuclear chromatin is shown after combined staining with propidium iodide/bisbenzimide (see Methods). Cells either untreated (CTRL) or treated with etoposide (100 μmol/l), (2-aminoethoxyethane)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (5 mmol/l) or ethylenediaminetetraacetic acid (EDTA) (5 mmol/l). Chromatin condensation or nuclei fragmentation are observed. Images were gathered under × 20 lens and × 5 digital zoom. Typical results from two experiments are shown.

EDTA used caused a dramatic drop in the population of viable cells at least after first 6 h of incubation (Fig. 1b, $P < 0.001$). The lower concentration of EGTA (2 mmol/l) did not diminish cell viability, whereas higher concentration of EGTA (5 mmol/l) exerted moderate cytotoxic effect (Fig. 1a, $P < 0.001$).

Calcium chelators differently affect apoptosis of COLO 205 cells

AI calculated for cultures treated with EGTA could not be faced up with EDTA. In contrast to EGTA, the average values of AI in EDTA treatments could not be determined as excessive fragmentation of detached cells occurred (Fig. 2). Additionally, both microscopic observations in ultraviolet light (Hoechst 33342) and in visible light (prior to MTT assay) confirmed the presence of massive cellular debris. For EGTA, the AI amounted to 1.18 ± 0.17, 4.16 ± 0.61 and 4.04 ± 0.57 for control, 2 mmol/l EGTA and 5 mmol/l EGTA, respectively. Besides, the etoposide induced apoptosis in COLO 205 cells in a dose-dependent manner. AI amounted to 1.95 ± 0.31, 11.01 ± 0.88 and 23.73 ± 0.93 for control, 50 μmol/l and 100 μmol/l of etoposide, respectively (Fig. 3a, $P < 0.001$). Apparently, both EDTA and etoposide evoked marked apoptogenic stimuli, although it is discussed below that they acted in a different way.

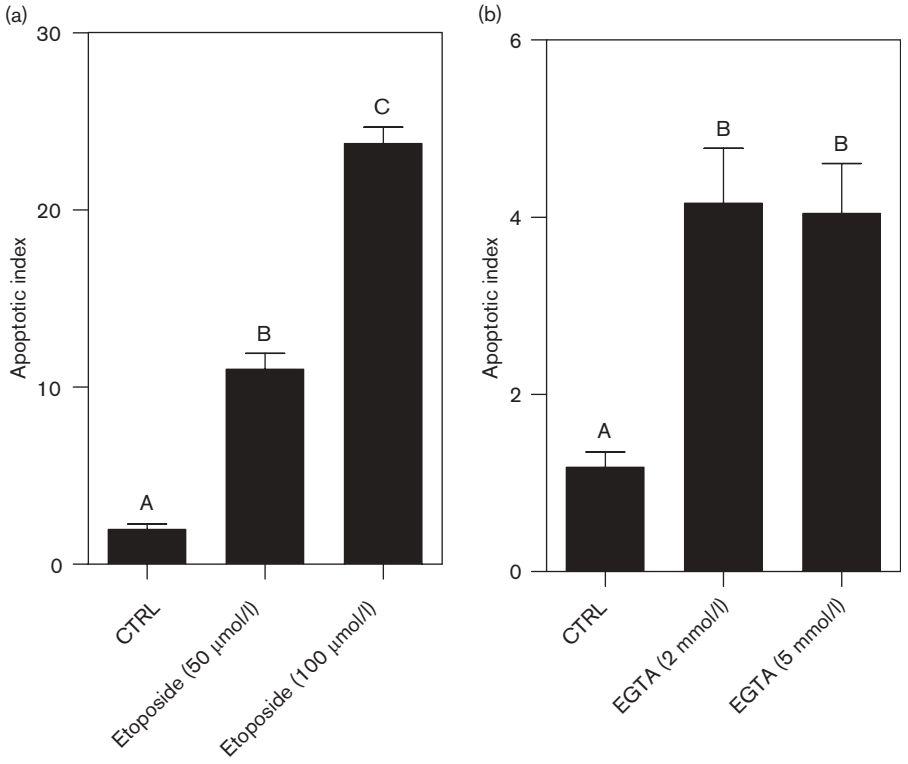
Etoposide induces nuclear expression of the 50-kDa intracellular isoform of clusterin protein in COLO 205 cells

According to Caccamo *et al.* [21] and Scaltriti *et al.* [15], the inhibitor of topoisomerase II, etoposide, is able to induce apoptosis with concomitant translocation of iCLU. As expected, the 24-h treatment with etoposide dose dependently (25, 50, 100 μmol/l) reduced cell viability measured by the MTT assay in COLO 205 cells (Fig. 4, $P < 0.01$). The Western blot analysis showed that etoposide-mediated cell death was accompanied by changes in iCLU subcellular level. Etoposide, in a dose-dependent manner, led to increased expression of iCLU in the nucleus, whereas no changes in the expression of cytoplasmic secretory CLU were observed. The latter is visible as the additional band of 40 kDa on the immunoblot (Fig. 5). Finally, etoposide dose dependently diminished procaspase-3 level in COLO 205 cells (Fig. 6)

Intracellular calcium limits nuclear expression of the 50-kDa intracellular isoform of clusterin in COLO 205 cells

Using the above experimental model, cells were then treated with 2 and 5 mmol/l of either EGTA or EDTA. As the EDTA-induced cell death was observed in the 6 h of

Fig. 3

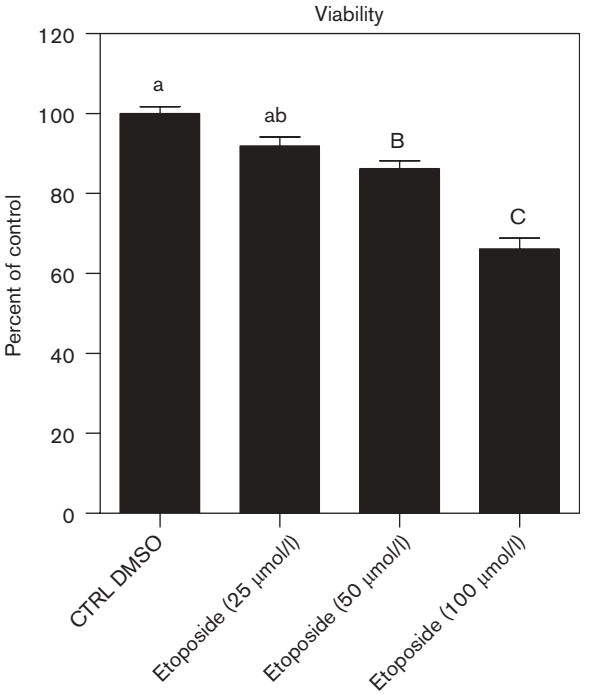


Bar charts representing apoptic indices calculated for etoposide (a) and (2-aminoethoxyethane)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (b). Different upper case letters indicate very highly significant differences between means. CTRL, control.

incubation, we chose that time for Western blot analysis to check the localization of iCLU in COLO 205 cells. All three isoforms of CLU, 60-kDa precursor, 40-kDa band (representing the 80-kDa secretory form) and intracellular 50-kDa CLU, were found. Similar to untreated cells, both concentrations of EGTA did not alter subcellular CLU distribution regardless of the CLU isoform, whereas EDTA at either concentration induced nuclear expression of the 50-kDa isoform of CLU (Fig. 7).

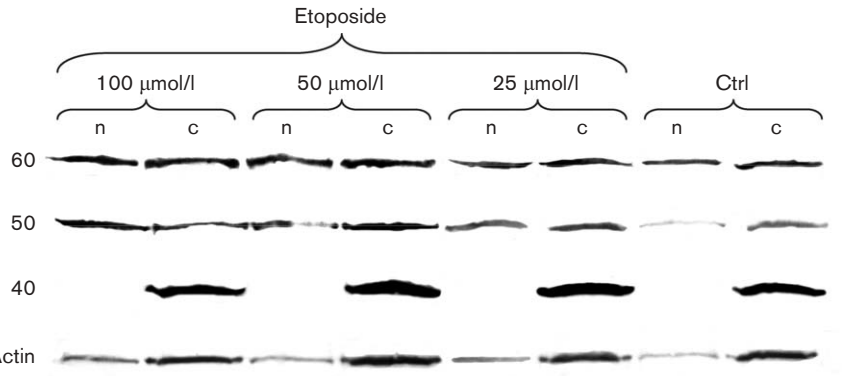
Etoposide treatment dose dependently reduced the viability of COLO 205 cells as demonstrated by MTT assay (Fig. 4, $P < 0.01$). According to previous studies on the COLO 205 cell line [30], we assumed that etoposide-induced reduction of COLO 205 cells viability was the result of apoptosis induction. Similar to prostate cancer cells, COLO 205 cell death was preceded by altered subcellular distribution of the proapoptotic 50-kDa isoform of CLU. We also noticed that the higher concentration of etoposide correlates positively with the higher level of iCLU in the nucleus (Fig. 5). As calcium homeostasis is suspected to be one of the modulators of CLU activity, we decided to deplete calcium by using calcium chelators. Previously, Chiesa *et al.* [31] had shown that extracellular calcium depletion reduces the expression of CLU mRNA. A recent study by Caccamo *et al.* [5], however, described the effect of calcium depletion on CLU proteomic profile. They demonstrated that in prostate PNT1A cancer cells, the intracellular chelation of Ca^{2+} with 1,2-*bis*-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra-acetoxymethyl ester (BAPTA-AM) led to the translocation of iCLU to the nucleus with the subsequent inhibition of cell proliferation followed by caspase-dependent apoptosis by anoikis. In our experiments, we also observed detachment of COLO 205 cells during EDTA treatment (data not shown) accompanied by the decreased cell survival and massive cell fragmentation (Fig. 1b and 2, $P < 0.001$). As it was indicated in prostate cancer, it is very likely that COLO 205 cells also died by anoikis. Similar to EDTA treatment, cell viability was reduced by the depletion of extracellular calcium ions by 5 mmol/l EGTA (Fig. 1a, $P < 0.001$). In contrast to EDTA, EGTA-induced cell detachment (anoikis) was, however, not observed. With regard to the COLO 205 cell line, these observations suggest that there are at least two distinct molecular mechanisms of low-concentration calcium-induced cell death. Apparently, the involvement of intracellular, but not extracellular calcium depletion is crucial for nuclear expression of CLU and nuclear CLU-dependent cell death of COLO 205 cells. To verify

Fig. 4



3-(4,5-Dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide assay of COLO 205 cells in the presence of etoposide (25, 50 or 100μmol/l for 24 h). Cell viability was calculated as a percentage of the control (CTRL) value. Bars ticked with different letters indicate significant (lower case, $P<0.05$) or highly significant (upper case, $P<0.01$) differences between the means. The studies were performed in quadruplicates and repeated at least twice with similar results. DMSO, dimethyl sulfoxide.

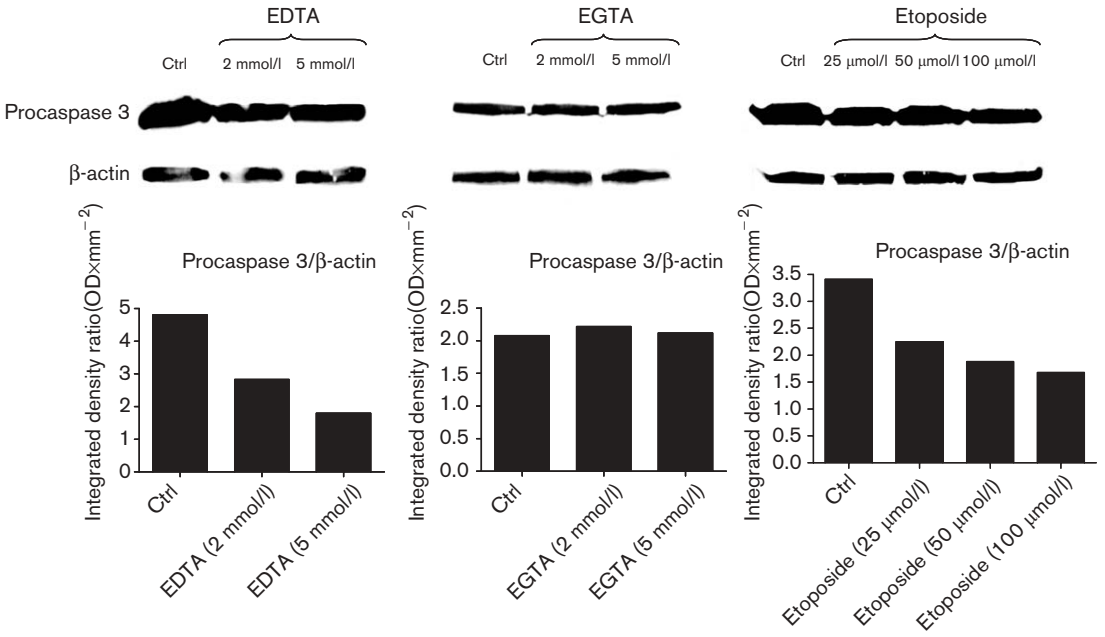
Fig. 5



Western blot analysis of clusterin (CLU) isoforms (60 kDa – precursor CLU, 50 kDa – intracellular CLU, 40 kDa – secretory CLU). The expression and localization of CLU isoforms after 24-h etoposide treatments (25, 50 or 100 μmol/l in culture medium) in nuclear (n) and cytoplasmic (c) fractions of cell lysates. Equal protein loading was checked by β-actin protein level. Each experiment was repeated at least twice with similar results. Ctrl, control.

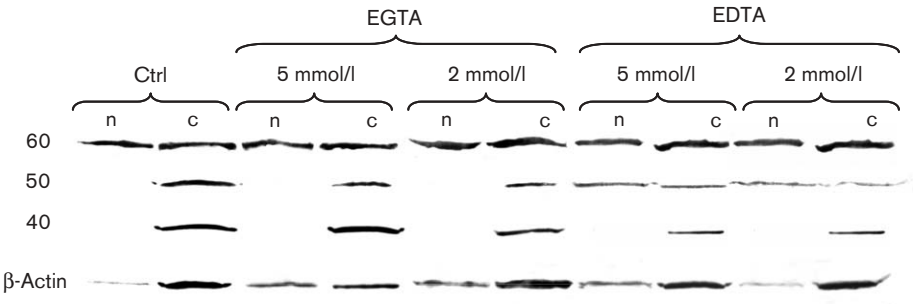
whether and how the level of calcium modulates the subcellular distribution of CLU, Western blot analysis was used. All CLU isoforms were identified in the cellular and nuclear fraction of COLO 205 cells treated with EGTA or EDTA, respectively (Fig. 5). To define the subcellular distribution of CLU, we focused our attention on 50 kDa iCLU isoform. In untreated cells, regardless of the concentration of EGTA, the 50-kDa CLU was present exclusively in the cytoplasm (Fig. 5). In contrast, the nuclear expression of 50-kDa CLU was stimulated by

Fig. 6



Western blot analysis of the whole-cell lysates of COLO 205 cells. The expression of procaspase-3 protein after 6 h of (2-aminoethoxyethane)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (2 or 5 mmol/l), ethylenediaminetetraacetic acid (EDTA) (2 or 5 mmol/l) or etoposide (25, 50 or 100μmol/l) treatments. Equal protein loading was checked by β-actin protein level. Bar charts below the respective blots represent relative integrated optical density values. Each experiment was repeated at least twice with similar results. Ctrl, control.

Fig. 7



Western blot analysis of clusterin (CLU) isoforms (60 kDa – precursor CLU, 50 kDa – intracellular CLU, 40 kDa – secretory CLU). The expression and localization of CLU isoforms after 6 h of (2-aminoethoxyethane)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (2 or 5 mmol/l) or ethylenediaminetetraacetic acid (EDTA) (2 or 5 mmol/l) treatments in nuclear (n) and cytoplasmic (c) fractions of cell lysates. Equal protein loading was checked by β-actin protein level. Each experiment was repeated at least twice with similar results. Ctrl, control.

EDTA administration. These results are in agreement with those reported by Caccamo *et al.* [5]. In their studies, however, CLU protein migrated to the nucleus in the presence of the BAPTA-AM, the intracellular calcium chelator. Instead of BAPTA-AM, we used EDTA, which chelates both the intracellular and extracellular calcium ions. As no nuclear CLU was observed after chelation of the extracellular calcium ions by EGTA, we conclude that it was the deficit of intracellular calcium ions that regulates apoptotic activity of CLU in COLO 205 cells. These data would also indicate that EGTA lowered the viability of COLO 205 cells by different and distinct molecular mechanism than did EDTA. It is very likely that a certain cytoplasmic concentration of calcium ion is essential for colorectal cancers to resist cell death induction. Interestingly, most of the published studies

demonstrate the 50-kDa clusterin translocation from cytoplasm to the nucleus during apoptosis. In our studies, the increased protein level within the nucleus was not correlated with the reduction of cytoplasmic protein level. We theorize that EDTA-induced cell death could activate changes in CLU gene transcription and lead to the synthesis of nuclear isoform of CLU. Additional studies are needed to identify the precise regulatory mechanism of CLU activity triggered by calcium ions. It would also be advantageous to determine how depletion of intracellular calcium led to nuclear expression of CLU that triggered apoptosis. These results indicate the possibility of redundant death pathways that might exist in COLO 205 cells and at least one of them could be activated by modulators of intracellular calcium. In summary, our results suggest that intracellular manipulation of calcium ion fluxes might be helpful in the control of life and death of cancer cells of colon origin, even those resistant to other types of death stimuli.

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