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Clusterin: the missing link in the calcium-dependent resistance of cancer cells to apoptogenic stimuli

Klasteryna – brakujące ogniwo w wyjaśnieniu zależności pomiędzy homeostazą wapniową a opornością komórek nowotworowych na apoptozę

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Summary

The resistance of cancer cells to artificially induced apoptosis comprises a major pitfall in contemporary chemotherapy. In recent years, a wide range of molecular mechanisms was revealed that allow cancer cells to overcome apoptosis. In prostate, breast, and colorectal cancers, a protein named clusterin was identified with anti- or proapoptotic activity regulated by calcium homeostasis. Reports so far suggest "two faces" of clusterin activity: the calcium-dependent cellular retention of clusterin positively correlates with cell survival, whereas nuclear translocation of this protein promotes cell death in calcium-deprived cells. Better understanding of the proprieties of clusterin isoforms and the molecular mechanisms which regulate its activity provide the prospect of breaking down cancer cells' resistance to apoptosis. Thus one might expect such dual benefits as overcoming the "immune escape" of neoplastic cells and reducing the doses of cytostatic drugs, with a concomitant reduction in the side effects of chemotherapy.

Key words: **clusterin • apoptosis • colorectal cancer • prostate cancer**

Streszczenie

Oporność komórek nowotworowych na apoptozę jest główną przyczyną niepowodzeń stosowanej obecnie chemioterapii. Badania kilku ostatnich lat ujawniły obecność wielu mechanizmów umożliwiających komórkom nowotworowym unikanie sygnału śmierci. W komórkach nowotworów prostaty, piersi oraz okrężnicy, zidentyfikowano białko – klasterynę, którego aktywność zarówno pro- jak i antyapoptotyczna są zależne od stężenia jonów wapnia. Dotychczasowe badania sugerują dwoisty charakter działania klasteryny. Obecność w środowisku komórki jonów wapnia determinuje cytoplazmatyczną lokalizację klasteryny, co dodatnio koreluje z żywotnością komórek i opornością na apoptozę. Z kolei ubytek jonów wapnia indukuje translokację jądrową białka, co w konsekwencji prowadzi do apoptozy. Poznanie właściwości różnych izoform klasteryny oraz mechanizmów regulujących aktywność każdej z nich wydaje się obiecującym narzędziem w przełamaniu niewrażliwości komórek nowotworowych na apoptozę. Pozwoliłoby to na zredukowanie dawek chemioterapeutyków używanych w leczeniu przeciwnowotworowym, a tym samym ograniczyłoby wynikające z niego działania niepożądane.

Słowa kluczowe: **klasteryna • apoptoza • rak okrężnicy • rak prostaty**

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Introduction

Today, the resistance of cancer cells to the natural mechanisms of cell elimination induced by the immune system as well as chemo- and/or radiotherapy causes a major problem. It is known that the primary induction of carcinogenesis is caused by the activation of oncogenes and the loss of tumor suppressor genes. As a result of changes in the gene expression profile, a number of other genes are upregulated. The final consequence appears as a dramatic disruption in physiological cell functions, including unrestricted proliferation, migration, and the formation of metastases. Therefore, the novel cellular "status quo" is a surplus of newly formed neoplastic cells that sustain tumor growth and render some cells immortal. Additionally, some of the newly achieved molecular mechanisms give the cancer cells the unique ability to avoid immune system attack. In principle, the presence of several novel molecular mechanisms allows cancer cells to escape the apoptosis induced by death ligands, such as TNFα, TRAIL, and FasL. Cancer cells often modify the ligand-induced signaling pathways to cease and/or counteract their respective outcomes. In colorectal cancer, which ranks third in terms of occurrence and death rate among populations of the United States and Western Europe, several cytoprotective mechanisms were identified, e.g. soluble death receptors, internalization of death receptors, decoy receptors, reverse signaling, and the release of anti-inflammatory cytokines and antiapoptotic proteins. Current efforts are focused on identifying antiapoptotic proteins, which could be a possible target for novel chemotherapeutic drugs. It has been demonstrated that, for example, FLIP protein is overexpressed in many cancer cells, also in colon cancers, and inhibits TRAIL-, FasL-, and TNFα-induced cell death by inhibition of caspase-8 activation. *Ex vivo* studies of colon adenomatous polyps and adenocarcinomas revealed that the intracellular concentration of FLIP is directly related to the grade of the tumor [36]. Moreover, *in vivo* studies of the TNFα-resistant human colon adenocarcinoma cell line COLO 205 confirmed the presence of FLIP protein and its interaction with the TNFα signaling complex [29]. Although the synthesis inhibition of short-lived antiapoptotic proteins by cycloheximide [11], sodium butyrate [15], bisindolylmaleimide [40], or other metabolic inhibitors sensitizes cancer cells to death ligand-induced apoptosis, it is often insufficient for the successful and complete elimination of tumors. In cancer cells, death signals are most likely frequently blocked downstream in the death receptor pathway. Among the antiapoptotic proteins blamed for this phenomenon, clusterin is a potent candidate. The exact function of clusterin in the regulation of cell death remains unclear, especially in cancer cells, although, according to the latest reports, clusterin could be a missing link between the inefficiency of chemotherapy and stimulation of the immune system to eliminate cancer cells.

The structure of clusterin: implications for cell death regulation

The major physiological function(s) of clusterin has not been clarified yet. It is known that clusterin (CLU), also called TRPM-2, SGP-2, XIP8 (ionizing radiation (IR) – induced protein-8), ApoJ (apolipoprotein J), SP 40-40, complement lysis inhibitor, gp80, glycoprotein III, and T64, is engaged in a number of biological processes [34,42]. Clusterin has been implicated in aggregating spermatozoa and sperm maturation [10], lipid transport, cell-cell and cell-matrix adhesions, cell differentiation, membrane recycling [22], cell membrane protection, and programmed cell death [19]. Interestingly, clusterin has been described both as a pro- [8,25,28,33] and as an antiapoptotic protein [23,26,27]. These contradictory reports could be related to the specific proteomic profile, which results from the complex expression of CLU. In spite of coding by a single gene located on human chromosome 8 [32], clusterin appears as different isoforms in the respective cell compartments. The main product of clusterin gene translation is a ~60-kDa protein called pre-sCLU (pre-secretory form). This form is produced by translation from the first AUG codon of the full-length CLU mRNA [25]. Pre-sCLU is transported from the polyribosome to the endoplasmic reticulum (ER) by the initial leader peptide [18] and then glycosylated and finally cleaved into α- and β-subunits, held together by five disulfide bonds [41,42]. The mature, ~80-kDa form of sCLU, highly conserved in different species, is secreted to the extracellular space and body fluids [18]. Aranow et al. [2] reported that following specific stress-induced injury, such as heat shock, sCLU acts as a molecular chaperone, scavenging denatured proteins outside the cells. This activity is brought about by the ability of nonspecific binding to the hydrophobic domains of various proteins [17]. Moreover, the results published by Araki et al. [1] showed that sClu secretion could also be caused by oxidative stress (ROS) following multiple organ dysfunction (MOD). Leskov et al. [25] demonstrated the presence of a second isoform of clusterin derived from an alternative splicing of the mRNA transcript. The product of the translation from the second AUG codon is a 50- to 55-kDa form of intracellular (iCLU), also called pre-nuclear CLU (pre-nCLU), which is localized in the cytoplasm. Upon some cytotoxic stimuli, including ionizing radiation (IR) [42], transforming growth factor β [33], or phorbol ester (TPA) [3], pre-nCLU (iCLU) is activated and translocated to the nucleus. nCLU lacks the leader peptide and

does not undergo glycosylation and α/β cleavage. Other alternative clusterin isoforms, produced by an exon skip induced by cell damage [21] or post-translational modifications, have also been described [24]. The vast majority of data suggests that nCLU contributes to cell death, whereas the form located in the cytoplasm (iCLU) does not exert apoptotic effect [25,31,42]. The first functional link between CLU and apoptosis was described when CLU was identified and cloned as a major product of mRNA transcription in prostate regression following surgical castration [35].

Nuclear clusterin interacts with Ku70/Ku80 complex

According to Yang et al. [42], nCLU is an important mediator of cell death after IR. Their studies revealed that IR induces CLU interaction with Ku autoantigen, a component of the DNA double-strand break (DSB) repair complex in the human breast cancer cell line MCF-7. They also found that the levels of both the precursor ~60-kDa and the secretory ~80-kDa CLU were considerably elevated in MCF-7 cells 2-4 days post-exposition to IR. Yeast two-hybrid analysis and co-immunoprecipitation studies revealed that only nonglycosylated nCLU forms complexes with Ku70 protein or Ku70/Ku80 heterodimer and does not interact directly with Ku80 protein. This interaction is mediated by the C-terminal coiled-coil domain of clusterin and the C-terminal domain of Ku70 [25]. In contrast, in nonirradiated cells inactive pre-nCLU was localized in the cytoplasm and did not bind to the Ku autoantigen complex. However, because the nCLU level increased 3 days after IR treatment and the repair of DBSs occurs within 2 hours post-treatment, the authors concluded that CLU is not engaged in DNA repair. Consistent with a previous study [43], they hypothesized that the CLU interaction with Ku70 suppresses Ku70/Ku80 DNA binding activity. The decrease in DNA-PK (DNA protein kinase) complex activity may be caused by caspase-mediated cleavage of the DNA-PK catalytic subunit, which correlates with the enhancement of CLU/Ku70 interaction [13]. Yang et al. [43] and Leskov et al. [25] showed that nCLU acts as a proapoptotic protein by inhibiting cell proliferation and survival and by promoting IR-resistant MCF-7 cells to IR-induced cell death. The stable transfection of MCF-7 cells and the increased level of nCLU resulted in cell lethality, even without IR treatment, whereas the overexpression of sCLU protein did not affect cell survival. These observations partially confirmed the results published by Redondo et al. [34]. The *ex vivo* studies on 140 invasive breast carcinomas showed that clusterin expression is related to tumor size and progression, whereas it is not detected in normal epithelial cells. However, immunohistochemical staining and *in situ* hybridization analyses identified overexpression and cytoplasmic localization of clusterin except for three cases of invasive carcinomas which had nuclear staining. Moreover, the increased clusterin expression in breast carcinomas correlated negatively with the apoptotic activity in these epithelia. The mean apoptotic index in clusterin-positive cells was lower (0.6) than in clusterin-negative tumors (1.1). In confronting these observations with Leskov's theory [25], we assume that the clusterin antiapoptotic function in breast carcinoma is associated with overexpression of intracellular clusterin and its retention in the cytoplasm. Consistent with this statement, the nuclear form of clusterin ultimately promoted cell death and in such circumstances the apoptotic index was increased. Thus, with respect to nuclei, clusterin-positive cells were more susceptible to apoptotic stimuli than clusterin-negative cells. This means that iCLU might modulate cell survival by this route. Similarly, Hara et al. [14] reported that overexpression of sCLU in human renal carcinoma cells enhances their resistance to chemotherapy and prevents apoptosis. However, the functional impact of the induction of iCLU in antiapoptos is still unknown. The data suggest that the transcription of one of the two mRNA transcripts is linked to the contemporary cellular state and could be influenced by factors such as cytokines, growth factors, or stress-inducing agents acting from the external milieu.

Clusterin is able to suppress NF-κB transcriptional activity

Irrespective of the postulated interaction of CLU with the Ku 70 complex, Santilli et al. [37] proposed another explanation for CLU's proapoptotic activity. They demonstrated that clusterin is involved in the regulation of NF-κB activity in the human neuroblastoma cell line LAN5. NF-κB is a transcription factor that plays an important role in the stimulation of cell survival and, in contrast to normal cells (except B cells), is constitutively expressed in many cancer cell types. The activity of NF-κB is suppressed through interaction with the inhibitory molecule IκB which, by ligation to p55/p60 heterodimer, blocks the translocation to the nucleus and its transcriptional activity. The phosphorylation of IκB by IκB kinases (IKKs) marks the latter for degradation, which results in the release and nuclear translocation of the active NF-κB heterodimer. Since about 300 genes, including those essential for antiapoptotic action, are under the control of NF-κB, the upregulation of NF-κB is a common trigger mechanism for the cell propagation essential for their transformation and tumorigenesis. Consistent with this hypothesis, a constitutively active IKK kinase significantly promotes invasion of neuroblastoma LAN5 cells [37]. According to these results, clusterin regulates NF-κB activity downstream of IKK kinase. The *in vitro* kinase assays established that clusterin does not inhibit IKK kinase activity, but extends the life-span of IκB protein. Moreover, the transfection of LAN5 cells with a specific NF-κB-responsive promoter, with or without a clusterin vector, showed 70% inhibition of the NF-κB-responsive promoter, suggesting that clusterin significantly suppresses NF-κB-dependent transactivation. Furthermore, deletion of the clusterin N-terminus, which contains the protein export signal, did not change its activity, indicating that the cytoplasmic rather than the secreted form is involved in the suppression of NF-κB activity. The exact mechanism of how clusterin regulates IκB protein turnover has not been described. It was shown that clusterin does not interact directly with IκB. The authors of this article [37] concluded that the effect could be achieved by direct regulation of IκB-interacting protein(s) or ubiquitin ligases. As a result of a proapoptotic clusterin-dependent suppression of NF-κB, the cancer cells may become more susceptible to growth inhibition and apoptogenic stimuli.

Clusterin and its role in prostate cancer tumorigenesis

The best-characterized models of tumor progression in prostate and colorectal cancer are also extensively studied

in the context of the role of clusterin in tumor progression [4,6,7,31,38]. Elucidation of the molecular mechanism leading to the androgen independence and apoptosis resistance of prostate cancer cells revealed the involvement of clusterin, especially of its 50-kDa isoform [5,38,44]. In experiments in which the cells were transfected with an expression vector containing the cDNA sequence for intracellular clusterin (according to Leskov et al. [25], starting from the second ATG codon on full-length clusterin cDNA), Scaltriti et al. [38] demonstrated that the cell survival of the PC-3 cell line is modulated by the cellular localization of the 50-kDa CLU. They also compared the consequences of transient and stable overexpression of the 50-kDa CLU. In PC-3 prostate cancer cells, transient overexpression of CLU caused a slight increase in the cell fraction present in the S and G2 phases of the cell cycle, followed by extensive cell death after 24 h, which became significant at 36 and 60 hours. At least 75% of the transfected cells showed nuclear clusterin localization. As a positive control for programmed cell death, etoposide, a well-known chemotherapeutic drug and inhibitor of topoisomerase II, was used. The effect of etoposide-induced cell death at 24 hours was very similar to the clusterin-induced apoptosis at 60 hours. Moreover, the decreased proliferation and accelerated cell death were caused by caspase-mediated apoptosis, because the administration of benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), a pan-caspase inhibitor, significantly reduced the proapoptotic activities of clusterin as well as etoposide [38]. In recombinant PC-3 cell clones exemplified by a stable overexpression of the 50-kDa CLU, the presence of a high level of CLU resulted in a reduction in colony formation compared with clones without CLU expression. In addition, the size and number of colonies were reduced. Similarly to the cells with transient CLU overexpression, a high level of CLU promoted cell death. In contrast, apoptosis was caspase independent and z-VAD-fmk did not modify the rate of cell death. In the colonies which, despite a high level of CLU, were able to survive, clusterin was identified in the cytoplasm. These observations are in agreement with Leskov's theory and suggest that PC-3 cells death was associated with the activity of the nonglycosylated form of nCLU, whereas inhibition of intracellular clusterin translocation to the nucleus was involved in avoiding apoptosis. Consequently, paclitaxel, a commonly used chemotherapeutic drug, was inefficient in inducing apoptosis in PC-3 cells [27]. The inhibition of translocation of the 50-kDa CLU to the nucleus and intranuclear CLU accumulation also account for the irradiation-resistant phenotype of PC-3 cells [44]. Moreover, IR treatment stimulates CLU expression, which in turn increases the resistance of PC-3 cells to apoptotic stimuli. Zellweger et al. [44] demonstrated that the CLU level in PC-3 cells increases even after a very low dose of irradiation. To confirm the crucial role of the 50-kDa CLU in cell death, specific antisense oligonucleotides were used to block clusterin gene expression. The inhibition of CLU expression, confirmed by Northern blot, caused cell cycle arrest and apoptosis. The authors concluded that targeted suppression of clusterin could increase the radiosensitivity of PC-3 cells. Caccamo et al. [5] presented interesting observations pointing to the role of CLU in anoikis, the apoptotic process of cell detachment induced by inappropriate cell-matrix interactions [9]. Besides loss of adhesion and cell shrinkage, anoikis shares the same morphological features of apoptosis as caspase activation, chromatin condensation, nuclear fragmentation, and apoptotic bodies [20]. It was also observed that cell detachment activates the expression of new genes. Since the clusterin gene was found as one of the genes whose activity was specifically suppressed by anchorage [12], its role in the anoikis of immortalized prostate cells has been determined. PNT1A cells were obtained by immortalization and cloning of normal human prostate epithelial cells stably transfected with SV40 large-T antigen. They maintain the expression of epithelial differentiation markers, such as cytokeratine 18 and 19. The cells routinely grow in RPMI medium containing FBS, whereas cultured in KSFM (keratinocyte serum – free medium) showed retarded proliferation and cell death. Clusterin was undetectable in the control cells (RPMI medium). Replacing RPMI medium with KSFM resulted in the expressions of the pre-sCLU 65-kDa and secretory 80-kDa isoforms in PNT1A cells after a 2-day culture. Concomitantly, secreted extracellular CLU was also found in the medium. The same cells after an 8-day incubation in KSFM showed an elevated level of the 45-kDa intracellular CLU, the form which was not secreted to the medium. The increasing level of the 45-kDa CLU during the following days was directly proportional to the rate of cell death, the type of which was additionally assessed by measuring the degraded PARP accumulation (the product of caspase 3 activity). In PNT1A cells, apoptosis was observed on the 8th day of KSFM incubation, whereas changes in the clusterin proteomic profile occurred as early as 2 days after changing the medium from RPMI. Moreover, the intracellular accumulation of the 45-kDa CLU was accompanied by a decrease in the CLU mRNA level, suggesting that this event was determined by post-translational protein modifications. Moreover, nCLU 45-kDa isoform accumulation and cell detachment occurred much earlier than the activation of caspases. The described changes in the protein profile were associated with different cellular localization of clusterin. The increasing level of nCLU was observed in the cell nucleus, and after 8 days of treatment nuclear accumulation was strictly associated with the disappearance of the 45-kDa intracellular CLU. In contrast, under RPMI-growing conditions, clusterin was constantly localized in the cytoplasm. To check whether the nuclear localization of nCLU was caused by KSFM treatment or it is also present in other models of apoptosis, etoposide was used, similarly to the Scaltriti et al. studies [38]. As expected, etoposide treatment was sufficient to induce a strong nuclear CLU localization signal accompanied by an increase in caspase-3 activity. To further assess the roles of clusterin in the apoptotic process, the same group studied the expression pattern during the regulation of calcium homeostasis in PNT1A prostate cells [4]. As described above, KSFM growing conditions caused cell death by anoikis. Interestingly, the addition of 1.8 mM Ca^{2+} or z-VAD-fmk, a caspase inhibitor, to KSFM completely rescued cells from apoptosis. Western blot analysis showed that both Ca^{2+} and z-VAD-fmk also blocked 65-kDa CLU conversion to the 45-kDa isoform and prevented its accumulation in the nucleus. Simultaneously, PARP fragments were undetectable. To elucidate whether Ca^{2+} depletion could activate the 45-kDa clusterin, the intracellular calcium ion chelator BAPTA-AM was used, resulting in a highly significant inhibition of cell growth, reaching about 78% after 48 h. This was associated with chromatin condensa-

tion and fragmentation and other hallmarks of anoikis. Similarly to the previous report, the changes in the clusterin proteomic profile appeared early (after 4 hours), but nCLU accumulation was the highest along with caspase activation (24 h). The presence of z-VAD-fmk or 1.8 mM Ca^{2+} showed that after 8 days in culture, clusterin was almost exclusively detected in the cytoplasm and the growth rate was similar to that of the control cells and higher than that observed in KSFM alone. Interestingly, changes in calcium homeostasis caused by the calcium chelator BAPTA-AM did not prevent sClu induction and did not modulate cell lethality after thapsigargin (TG) or IR treatment [1]. Taking these observation together, it is concluded that calcium deprivation causes iCLU activation and accumulation in the nucleus, leading to cell death by anoikis. All the presented data clearly show that in prostate cancer cells, clusterin plays an important role in cell survival. In line with Leskov's theory, the presented observations concerning intracellular clusterin, cytoplasmic localization and accumulation, confirmed its antiapoptotic function. Activated by various stimuli, including intracellular Ca^{2+} depletion, it translocates to the nucleus and induces cell death.

Clusterin and colon cancer progression

In like manner, *in vitro* and *in vivo* studies of colorectal cancer also demonstrated a decreased level of nuclear clusterin which is directly related to increased cell survival, aggressiveness, and enhanced metastatic potential. Northern blot analysis with clusterin cDNA revealed a highly elevated level of clusterin in tumor-bearing gut compared with normal subjects [7]. Subsequent *in situ* hybridization and immunohistochemistry also showed a strong clusterin signal present in tumor tissues. Accordingly, in immunohistochemical studies presented by Pucci et al. [31] and in immunofluorescence assays [7], clusterin expression and localization were related to tumor stage and grade. For example, in the colonic mucosa of healthy subjects and in normal mucosa apart from the neoplasia, clusterin was mainly localized in the nucleus, whereas in the cytoplasm CLU was almost undetectable. In more advanced tumor stages, clusterin was detected only in the cytoplasm. Moreover, clusterin was found to be overexpressed in highly aggressive and metastatic tumors. In these tumors, clusterin was also released to the extracellular space. These observations were confirmed by Western blot analysis, which showed a variety of clusterin isoforms. The expression of proapoptotic nCLU was evident in normal mucosa and was completely absent in all the tumor tissues. Hence, the 40-kDa clusterin form, corresponding to the secreted form, was present both in normal and in tumor samples, while in invasive carcinomas it was additionally overexpressed (the level of sCLU was 1.5- to 4-times higher than in normal counterparts) [31]. Chen et al. [6] presented similar results. A low level of clusterin was detected in the cytoplasm of normal colonic epithelial cells, whereas the cytoplasmic localization of CLU was elevated in hyperplastic polyps, tubular adenomas, and villous adenomas. In the most advanced colonic tumors (invasive adenocarcinoma), immunohistochemical staining demonstrated the presence of CLU also in the intracellular spaces. Interestingly, the morphologically normal cells close to the tumor cells also showed a clusterin signal in their cytoplasm. The authors did not explain this phenomenon. It has not yet been established if sCLU present in the extracellular space has the ability to induce CLU expression or to enter other cells. In both reports the correlation between clusterin and apoptosis was also determined. The fluorescent TUNEL assay, which stains cells undergoing apoptosis with DSBs, showed negative correlation between clusterin mRNA and the apoptotic index in tumors [6,31]. Moreover, Pucci et al. [31] performed *in vitro* experiments on the Caco-2 cell line to determine whether the expression of the nuclear proapoptotic clusterin form, completely lost in highly aggressive tumors, is involved in apoptosis induction. They evaluated the presence of different isoforms of clusterin in cells treated with the cytostatic hormone somatostatin (SST). Western blot analysis showed that a 4-hour incubation with SST caused a strong decrease in the 50-kDa CLU in the cytoplasm with a concomitant increase in this form within the nucleus. A similar experiment on freshly isolated colon cells from a human low-grade carcinoma fully confirmed these results. Furthermore, the glycosylation assay revealed nuclear localization of the nonglycosylated nCLU isoform after SST treatment. Analogous results have been presented by Chen et al. [6]. After treatment with chemotherapeutic compounds of the HT 29, HCT 116, and KM12C colon cell lines, apoptosis was induced and was directly related to the increased level of nCLU. Clusterin protein was not detected in the SW620 cell line, which is resistant to chemotherapy-induced apoptosis. Chen et al. [6] also showed that clusterin in transiently transfected HT29 cells induces apoptosis in >30% of cells compared with mock transfected cells. All these observations confirmed the contribution of the 50-kDa nCLU to the induction of cell death in colon carcinomas. The overexpressed CLU isoform in Caco-2 cells, similarly to immunohistochemically stained tumor, was identified as a secreted form of CLU. To date, reports indicate that the activity of proapoptotic nCLU could be modified in human and mice colon cancer cells by *APC* gene [6,7]. Lack of a functional *APC* gene results in tumorigenesis and resistance to apoptotic stimuli [16,30]. According to this hypothesis, the level of nCLU in regular cells should be diminished compared with $APC^{+/+}$ cells. Chen et al. [7] demonstrated that in a null $APC^{-/-}$ human cancer cell line (HT-29) stably transfected with inducible wild-type *APC* vector, the clusterin mRNA level dramatically increased after induction of the wild-type *APC* expression. Moreover, the use of antisense oligonucleotides blocking clusterin gene expression resulted in a 50% decrease in apoptotic cells. These results indicate that APC-induced colon cancer cell death is dependent on clusterin expression. In contrast, immunohistochemical staining and *in situ* hybridization in various human colorectal tumors allowed identification of clusterin in $APC^{-/-}$ cells accumulating β-catenin protein in their cytoplasm [7]. The authors did not determine which CLU isoform was present in the cells. We suppose that retention of the 50-kDa CLU in the cytoplasm, similarly to prostate cancer cells [38], caused the above-mentioned effect. As chemotherapeutic agents or APC enable up-regulating the cyclin-dependent kinase inhibitor p21, such treatment also induced clusterin expression. Chen et al. [6] examined whether correlation between CLU and p21 occurs. They used the wild-type HCT116$p21^{+/+}$ and knockout HCT116$p21^{-/-}$ cells. Treatment with a chemotherapeutic agent (5-FU or irinotecan) caused both clusterin and p21 expression in HCT116$p21^{+/+}$ cells. In cells without p21, the same treatment did not induce CLU expression. Moreover, HCT116$p21^{-/-}$ cells were more resistant to 5-FU and FasL-induced apoptosis than the parental

cell line. On one hand the authors concluded that unless p21 is overexpressed, clusterin expression cannot be induced and apoptosis is inhibited. On the other hand, transfection of CLU to the knockout cells was sufficient to induce nuclear CLU localization and cell death. To confirm the interaction of CLU with p21, the apoptosis-resistant SW620 cell line was used. As described previously, the SW620 cell line does not express clusterin and did not undergo chemotherapeutic drug-mediated apoptosis. Appropriate studies showed that these cells have no detectable p21 or clusterin expression regardless of the chemotherapeutic drugs used. Summing up, these results suggest that clusterin acts downstream of p21 and that the CLU overexpression after chemotherapy is p21 dependent. Identical studies questioned the implication of p53 in CLU proapoptotic activity. The details of the p21 signaling pathway that regulates nCLU activity remain unknown. In prostate cancer cells it was also demonstrated that p21 limits cell growth arrest in the G1 and G2 phase by inhibition of cyclin B1/cyclin-dependent kinase 1. This arrest is accompanied by apoptotic cell death. However, the exact mechanism of how p21 and CLU function in colon cancer cells needs to be explained. The question arises whether the cellular CLU accumulation is a cause or an effect of the triggered cell death machinery. It is postulated that the iCLU retention could be the outcome of interactions with other proteins localized in the cytoplasm. However, such an interaction was described for nCLU in the nucleus [39]. The cDNA microarray showed upregulation of the novel protein in colon cancer HCT116 cells. Because of its homology to the clusterin structure and, in accordance with the results obtained from the yeast two-hybrid screening system, it was called CLUAP1 (clusterin – associated protein 1) from its direct interaction with nCLU. This association was also confirmed by immunoprecipitation studies. The performed cell-cycle analysis and immunoblotting revealed synchronized upregulation of the nCLU and CLUAP1 levels, which abrogated cell death. It was demonstrated that the colonies transfected with nCLU alone and with nCLU and CLUAP1 show a similar reduction in the number of cell colonies. Consequently, the suppression of nCLUAP1 resulted in a decrease in the colony number of cells, suggesting that a decreased level of nCLUAP1 leads to growth suppression of the cancer cells tested. Perhaps the lower nuclear CLUAP1 localization simultaneously correlated with an increased level of this protein in the cytoplasm. However, the described report shows that unknown cellular proteins could modify clusterin's anti- or proapoptotic activity.

Summary

The changes in clusterin expression in breast, prostate, and colon cancers were described. Previous reports which presented conflicting results pointed to both pro- or antiapoptotic activity of clusterin. However, newer investigations seem to shed more light on this phenomenon. The observed effects of clusterin were specific to the recently characterized CLU isoforms. The cytoplasmic retention and high level of the 50-kDa CLU protect tumor cells from apoptotic stimuli induced by chemotherapeutic drugs or natural ligands, such as FasL [5,6,38], whereas its nuclear presence (nCLU) enhances cell apoptosis [25,42]. The 50-kDa clusterin isoform is mainly overexpressed in cancer cells and retained in the cytoplasm, promoting cancer progression and aggressiveness. Cytoplasmic clusterin could be easily translocated to the nucleus in the presence of various inducers, such as IR, chemotherapy [4,5], hormones [31], or cytokines, or because of cellular calcium depletion [4]. Apparently, the induction of nCLU seems to be tissue specific since the same treatment frequently induces rather different cellular responses. In addition, cells might tolerate clusterin overexpression (eventually provoked by proapoptotic signals) if rescuing factors can counterbalance clusterin action or if such cells have acquired a phenotype that is resistant to apoptotic stimuli. Bearing this in mind, the knowledge of clusterin activation could be a promising therapeutic perspective. Its proapoptotic activity is observed on different, independent levels: suppression of NF-κB activity, inhibition of antiapoptotic proteins, and inhibition of DNA repair mediated by reducing Ku autoantigen DNA binding activity. We predict that clusterin could be engaged in the escape of cancer cells from apoptosis induced by the natural death ligands TRAIL, FasL, and TNFα, because the proapoptotic clusterin translocation from the cytoplasm to the nucleus was observed in the FasL-sensitive cancer cells HT 29, HCT116, and KM12C, but not in death ligand-resistant cell lines [6]. Intriguing results were also obtained from experiments performed with calcium chelators. They all pointed to the importance of calcium homeostasis for clusterin activity. Combined treatment with calcium channel modifiers and radiotherapy or chemotherapy could sensitize cells to extracellular death ligand-induced apoptosis and render a more efficient deletion of cancer cells. Determining the signaling pathway of clusterin activation and its possible implications for cell survival could give more a rational perspective to the future prospects in anticancer therapy.

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