Cell detachment and apoptosis induction of immortalized human prostate epithelial cells are associated with early accumulation of a 45 kDa nuclear isoform of clusterin

Alessandro E. CACCAMO*, Maurizio SCALTRITI*, Andrea CAPORALI*, Domenico D'ARCA†, Francesca SCORCIONI†, Serenella ASTANCOLLE‡, Massimo MANGIOLA‡ and Saverio BETTUZZI*¹

*Dipartimento di Medicina Sperimentale, University of Parma, Via Volturno 39, 43100 Parma, Italy, †Dipartimento di Scienze Biomediche, Università di Modena e Reggio Emilia, Modena, Italy, and ‡Dipartimento di Oncologia, Biologia e Genetica, Università di Genova, Genova, Italy

Clusterin, ubiquitously distributed in mammals, was cloned and identified as the most potently induced gene during rat prostate involution following androgen deprivation. Also found to be involved in many other patho-physiological processes, its biological significance is still controversial, particularly with regard to apoptosis. We previously showed that transient over-expression of clusterin blocked cell cycle progression of simian-virus-40-immortalized human prostate epithelial cell lines PNT1A and PNT2. We show in the present study that the accumulation of an intracellular 45 kDa clusterin isoform was an early event closely associated with death of PNT1A cells caused by cell detachment followed by apoptosis induction (anoikis). Cell morphological

changes, decreased proliferation rate and cell cycle arrest at G₀/G₁–S-phase checkpoint were all strictly associated with the production and early translocation to the nucleus of a 45 kDa clusterin isoform. Later, nuclear clusterin was found accumulated in detached cells and apoptotic bodies. These results suggest that a 45 kDa isoform of clusterin, when targeted to the nucleus, can decrease cell proliferation and promotes cell-detachment-induced apoptosis, suggesting a possible major role for clusterin as an anti-proliferative gene in human prostate epithelial cells.

Key words: anoikis, apoptosis, caspase, clusterin, PNT1A cell, prostate cancer.

INTRODUCTION

Clusterin is a glycoprotein widely distributed in mammalian tissues and biological fluids [1] that is encoded by a single copy gene located on chromosome 8 in humans [2,3]. It was previously cloned and identified by different research groups working in widely divergent areas, because of its involvement in many biological processes [1,4,5]. The secreted heterodimeric form of clusterin is targeted to the endoplasmic reticulum as an initial precursor peptide of 65–70 kDa [6], which, after disposal of a 22 amino acid leader peptide, is then glycosylated and cleaved into one α - and one β -chain of about 40 kDa each, held together by a unique five disulphide bond motif [6]. It has been suggested that this secreted form of clusterin may act as an extracellular molecular chaperone [7], scavenging extracellular misfolded proteins that can be produced following stress-induced injury, such as heat shock [8]. Thus a role for extracellular clusterin in the clearance of cellular debris and phagocytosis promotion was suggested [9]. Beside its secreted mature form, the existence of a truncated isoform of the protein has been reported. This truncated protein was suggested to be targeted to the nucleus, acting as a pro-death signal by inhibiting cell growth and survival [10–12]. Other alternative clusterin isoforms, produced by exon skip induced by cell damage [13] or post-translational modifications activated by apoptosis-induced stimulation of a normally under-utilized synthetic pathway, have also been described [14]. The fact that clusterin was found involved in a plethora of patho-physiological processes, including cell adhesion and cell–cell interactions [1] or cancer, proliferation and aging [15–18], makes

this topic very intriguing. It is well known that clusterin gene expression is potently induced during rat prostate involution. This was demonstrated following surgical castration [5], chemical androgen ablation [19] and non-hypercalcaemic calcitriol-analogue treatment [20]. Its expression level also depends on the proliferation state, since it was found up-regulated in human fibroblasts when synchronized in the quiescence state [16] and down-regulated after induction of cell proliferation [16]. A causative role for this protein in regulating cell growth of prostate epithelial cells was recently suggested, after the finding that clusterin was shown to block cell cycle progression of simian virus (SV)40-immortalized human prostate epithelial cell lines PNT2 and PNT1A [21]. Nevertheless, the precise physiological role of clusterin has not been established yet. Some authors have proposed an anti-apoptotic role for clusterin [9,22,23], but others have found evidences in favour of pro-apoptotic functions [11,24,25]. More results are needed for the definition of this topic.

Survival of most normal epithelial cells requires adhesion to basement membrane, a special kind of extracellular matrix. Loss of cell–extracellular matrix contacts results in death of such cells by apoptosis, a phenomenon known as anoikis [26–28]. Anoikis, first documented in both epithelial and endothelial cells [26,27], and recently also in prostate cancer cells [29], is defined as an apoptotic process that is induced by inappropriate cell–matrix interactions [28]. Besides loss of adhesion, cell rounding and shrinkage, anoikis shares the same morphologic features and cell signal transduction pathways of apoptosis, including activation of genes such as caspases as effectors (e.g. caspase-3) [27,28], cytoplasm and chromatin condensation and nuclear fragmentation

Abbreviations used: DAPI, 4,6-diamidino-2-phenylindole; DEVD-pNA, Asp-Glu-Val-Asp-p-nitroanilide; FAK, focal adhesion kinase; FBS, foetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KFSM, keratinocyte serum-free complete medium; p-FAK-Tyr³⁹⁷, phosphorylated FAK; PAR, poly(ADP-ribose); PARP, PAR polymerase; pNA, p-nitroanilide; SV40, simian virus 40; TTBS, Tris-buffered saline/Tween.

¹ To whom correspondence should be addressed (email saverio.bettuzzi@unipr.it).

[30], finally leading to cell fragmentation into membrane-bound apoptotic bodies that are ingested by neighbouring phagocytic cells [31]. It has been suggested that a possible cause of anoikis might be the changes occurring in cell shape by alteration of cytoskeleton [32], leading to cell detachment and caspase-dependent cleavage of proteins assembled in focal adhesion complexes, involving FAK (focal adhesion kinase) [33]. Despite the increasing interest in the study of anoikis, the basic mechanisms of this phenomenon are still poorly understood. However, insights concerning apoptotic death of prostate cancer cells have been described [34]. To this end, it must be stressed that clusterin was found as one of the few genes specifically suppressed by anchorage [35].

It is known that apoptosis can be induced upon serum starvation in many cell types, including PNT1A cells. We observed that these cells, routinely grown in RPMI medium supplemented with FBS (foetal bovine serum) [36,37], showed decreased proliferation when grown in KSFM (keratinocyte serum-free complete medium) medium. KSFM medium is well known in the prostate field, because is routinely used for selection and maintenance of primary cultures of human prostate epithelial cells [38]. Conversely, PNT1A cells can be cultured in KSFM supplemented with FBS without any major adverse effect. Thus we studied clusterin expression in PNT1A cells grown in KSFM without FBS supplementation, an experimental model that can be considered an example of apoptosis induction by serum-free condition. This experimental approach was chosen in view of our interest in investigating the possible connection between clusterin and apoptosis. Thus we have studied the time-course of clusterin molecular changes in strict correlation to apoptosis induction to collect more experimental evidence for understanding the possible role of this gene in the apoptotic death of prostate epithelial cells.

EXPERIMENTAL

Cell lines and growth conditions

PNT1A cells were previously established [36,37] and characterized [39] with respect to morphological features, cytokeratins expression profile and doubling time under standard growth conditions [36,37]. PNT1A cell line was 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 cytokeratin 18 and 19, prostate specific antigen, prostatic acid phosphatase and androgen receptor, thus being androgen-dependent cells. Their proliferative response to growth factors has been studied previously [39]. Cells were cultured under standard conditions, with serum-fed medium RPMI 1640 supplemented with 10 % (v/v) FBS and 1 % (v/v) L-glutamine [36], or with the medium that is used for growing normal human prostate epithelial cells in primary culture, such as KSFM supplemented with 5 ng/ml epidermal growth factor, 50 µg/ml bovine pituitary extract and 1 % (v/v) L-glutamine. In both cases, cells were cultured without antibiotics at 37 °C in a 95 % air/5 % CO₂ atmosphere. Cell harvesting was performed routinely by trypsin/EDTA treatment. Culture media were purchased from Gibco Laboratories (Grand Island, NY, U.S.A.).

Cell viability and cell growth assays

Cell growth was assessed by the Crystal Violet method [40]. Briefly, adhering cells were fixed, stained with Crystal Violet and then the dye was extracted and the D_{540} was determined. After seeding cells in triplicate under the different experimental conditions studied, the culture medium was removed and cells

fixed with 4 % (w/v) paraformaldehyde in PBS for 15 min and stained with 0.1 % (w/v) Crystal Violet (Sigma, St. Louis, MO, U.S.A.) in 10 % ethanol for 20 min at 22 °C. Cells were then rinsed four times. Crystal Violet accumulated by adherent cells was then extracted with 100 µl sodium citrate 0.1 M (pH 4.2)/ethanol (2:1, v/v, final pH 5.2) and the D_{540} was determined by spectrophotometry. Attenuances are expressed as the means $\pm$ S.D. from 3 independent experiments (see Figure 1A). In parallel, cell viability was studied using the WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate} reagent method (Roche, Lewes, East Sussex, U.K.) according to the manufacturer's protocol. Briefly, after seeding cells in triplicate in 96-well plates under RPMI or KSFM conditions for the indicated times, cells were incubated for 1 h at 37 °C in a 95 % air/5 % CO₂ atmosphere in the presence of 10 % (v/v) of the tetrazolium salt WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate}. The number of viable cells were estimated on the basis of their ability to metabolize the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases. Then 10 % (v/v) SDS was added to denature the cells and extract the formazan dye. D_{450} was measured in SDS extracts by means of a scanning microplate reader (ELISA reader) MRX II (Dinex Technology). Attenuance values directly correlates with the number of metabolically active cells. The values shown in Figure 1(B) are the means $\pm$ S.D. from 3 independent experiments in comparison with time-matched standard RPMI controls.

Cell viability was also determined in parallel by counting the number of viable cells with the Trypan Blue dye exclusion test. Briefly, cells adhering to plates were quickly removed by trypsinization. Trypsin action was blocked by addition of medium, and cells were centrifuged at 250 g for 5 min. Cells were then recovered, suspended in RPMI medium with 0.1 % (v/v) Trypan Blue (Sigma) for 5 min and counted in a haemocytometer. Viable cells appear clear under the microscope, because of their capability to exclude Trypan Blue from the cytosol, while dead cells appear blue, because, having lost membrane integrity, they are unable to exclude the dye. Viability was determined as the percentage of viable (white) cells over the total number of cells (white + blue) at the indicated times. Three different fields containing at least 150 cells were randomly chosen for each experimental point from 3 independent experiments (results not shown). The cell viability data obtained by WST-1 and Trypan Blue dye exclusion test methods gave comparable results.

Northern blot hybridization analyses

Total RNA was extracted as previously reported [17], and 10 µg aliquots were electrophoresed and blotted on to nylon membranes (Zeta-Probe GT genomic membrane, Bio-Rad, Milan, Italy). Northern hybridization was performed using human clusterin, histone H3 (specific marker of the S-phase of the cell cycle) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase, a house-keeping gene) full-length cDNAs probes as described previously [15,17].

Western blot analyses

Cell population was harvested in RIPA extraction buffer [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % (w/v) sodium deoxycholate, 1 % (v/v) Triton X-100, 0.1 % (w/v) SDS, protease and phosphatase inhibitors cocktail (Sigma)] at 4 °C for 15 min. Then cell extracts were cleared by centrifugation at 14 000 g for 20 min at 4 °C and the supernatant was saved for protein determination by the dye-fixation Bradford method

(Sigma) using BSA as standard. The absorbance was measured at 595 nm. Then total protein extracts equivalent to 50 µg of protein/lane were heated at 100 °C for 5 min in SDS/PAGE loading buffer (1:1, v/v) under reducing conditions, resolved by electrophoresis under reducing conditions using a 10 % or 12 % (w/v) polyacrylamide gels (depending on the molecular mass of the protein to be detected), and finally blotted on to the PVDF membranes (Millipore SpA, Milan, Italy). After the blocking of non-specific binding sites with 1 % (w/v) non-fat dried milk blocking solution in TTBS [Tris-buffered saline/Tween 20: 100 mM Tris/HCl, pH 7.6, and 0.9 % (w/v) NaCl, 0.05 % (v/v) Tween 20, (Roche Diagnostics, Milan, Italy)] for 1 h at 22 °C, Western blot analyses were performed on the membranes by the chemiluminescence method as described previously [21]. Clusterin immunoreactive bands were detected with the BM Chemiluminescence Blotting Substrate (POD) (Roche Diagnostics) using mouse monoclonal anti-(human clusterin) antibody (clone 41D) from Upstate Biotechnology (Lake Placid, NY, U.S.A.; dilution, 1:1000). Then horseradish peroxidase-conjugated anti-mouse secondary antibody (Sigma) was used at 1:5000. For better comparison of p-FAK-Tyr³⁹⁷ (phosphorylated FAK) and FAK, both proteins were detected on the same blots. The membranes were stripped by incubation at 22 °C for 1 h in stripping solution (1 M glycine/1.5 M NaCl, pH 2.5, and 2.5 % SDS, w/v). Then the blots were washed six times for 10 min in TTBS, blocked again with non-fat dried milk blocking solution and re-probed with the appropriate antibody. Rabbit anti-(human p-FAK-Tyr³⁹⁷) and anti-(human FAK) (both at 1:1000 dilution; Upstate Biotechnology) were used. Then horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma) was added at 1:5000 dilution. For detection of PAR [poly(ADP-ribose)] attached to the proteins, the membranes were stripped as reported above and re-probed with mouse monoclonal anti-(human PAR) antibody (dilution 1:250; Oncogene Research Products, San Diego, CA, U.S.A.). Then horseradish peroxidase-conjugated anti-mouse secondary antibody was used at 1:5000 dilution. Finally, equal protein samples loading and transfer efficiency on the membranes were routinely monitored by Western blot analysis of cytokeratins expression on each membrane after stripping the blots and blocking non-specific sites. Mouse monoclonal anti-(human pan-cytokeratin) (dilution 1:100 000) antibody was used. In addition, 0.1 % (w/v) Red Ponceau S staining of the membranes was also performed. Pan-cytokeratin antibodies and Ponceau S were purchased from Sigma.

Preparation of subcellular extracts

Cells were lysed at 4 °C with 5 vols of buffer A [25 mM Tris/HCl, pH 7.4, 10 mM KCl, 0.15 % (w/v) β-hexyl glucopyranoside and protease inhibitor cocktail], and centrifuged at 8000 g for 10 min at 4 °C. The first supernatant was collected and stored at 4 °C, while the pellet was dissolved in two volumes of buffer A and centrifuged again at 8000 g for 10 min at 4 °C. Then, the second supernatant, containing the cytoplasm fraction, was collected. The two supernatants were then mixed gently together, added to 1 vol. of SDS/PAGE loading buffer, heated at 100 °C for 5 min, resolved by electrophoresis under reducing conditions on a 10 % (w/v) polyacrylamide gel and finally used for Western blot analysis (cytoplasm extract). The pellet was dissolved in 3 vols of buffer B [50 mM Tris/HCl, pH 7.4, 75 mM NaCl, 25 mM KCl, 5 mM MgCl₂, 0.5 % (v/v) Triton X-100 and 0.25 % (w/v) sodium deoxycholate] and centrifuged at 13 000 g for 10 min at 4 °C. The third supernatant, containing the mitochondria and the non-nuclear membrane fractions, was collected, and stored at -80 °C. The second pellet, containing the nuclear protein fraction, was

recovered, added to 1 vol. of SDS/PAGE loading buffer, heated at 100 °C for 5 min and resolved by electrophoresis under reducing conditions on a 10 % (w/v) polyacrylamide gel before being used for Western blot analysis (nuclear extract).

Detection of apoptosis and apoptotic bodies

Apoptosis rate in cell cultures was assessed both by caspase-3 activity determination and morphological analysis. Caspase-3 assay was performed by spectrophotometric determination of chromophore pNA (p-nitroanilide) released in solution after specific cleavage of the labelled substrate DEVD-pNA (Asp-Glu-Val-Asp-pNA) by caspase-3. The CPP32/Caspase-3 Colorimetric Protease Assay kit (MBL, International Corporation, Watertown, MA, U.S.A.), was used according to the manufacturer's instructions. Under standard growth conditions, KFSM treatment or etoposide administration, adherent plus floating cells (that detached from plates) were collected together, added to lysis buffer [10 mM Tris/HCl, pH 7.5, 10 mM NaH₂PO₄/Na₂HPO₄, pH 7.5, 130 mM NaCl and 1 % (v/v) Triton X-100], and left on ice for 10 min. The supernatant was then recovered by centrifugation at 12 000 g for 2 min at 4 °C. The equivalent of 50 µg of protein of the supernatant (cytosolic extract) was added to 50 µl of reaction buffer (1.4 M NaCl, 27 mM KCl, 100 mM KH₂PO₄/K₂HPO₄, pH 7.5 and 10 mM dithiothreitol) containing 200 µM (final concentration) of the fluorogenic substrate DEVD-pNA and then incubated at 37 °C for 2 h. Finally, the caspase-3 activity of the supernatant solution was measured at an A₄₀₅ by means of a scanning microplate reader (ELISA reader, MRX II, Dinex Technology).

Morphological analysis of apoptotic bodies was performed as follows: controls and treated adherent cells were fixed with 4 % (w/v) paraformaldehyde in PBS for 15 min and washed twice with PBS. Then cell nuclei were stained with 5 µg/ml DAPI (4,6-diamidino-2-phenylindole) or 0.5 % (v/v) Giemsa (both purchased from Sigma) for 10 min at 22 °C and mounted on to slides with 70 % (v/v) glycerol in PBS to visualize nuclear fragmentation and apoptotic bodies. Both methods allow the detection of chromatin condensation, nucleus and DNA fragmentation, all well-known indicators of apoptotic cell death. Nuclear morphology was analysed with a fluorescence microscope (Axio Vert 200, Carl Zeiss, Gottingen, Germany) and digital images were acquired with a colour digital camera (Axio Cam MR, Carl Zeiss) directly through the microscope. For comparison, DAPI nuclear staining was measured in parallel in both adherent and floating control or KFSM-treated cells by adding the fluorescent dye directly to the cell culture medium for 10 min at 37 °C. For quantification of apoptosis, positively stained cells were scored as normal or apoptotic on the basis of cellular morphology. Cells exhibiting membrane, cytoplasmic and nuclear condensation were scored as apoptotic cells. Three randomly chosen fields for each experimental condition containing at least 150 cells from 3 independent experiments were used, and data were calculated as the percentage of apoptotic figures over the total number of cells at the indicated times, and expressed as the means ± S.D., in comparison with time-matched standard RPMI controls.

Detection of DNA fragmentation and cell cycle progression analysis

Cell cycle progression was studied with the monoparametric FACS analysis method by means of propidium iodide staining for total DNA content as previously described [21]. Briefly, both floating and adherent cells were harvested and pooled together or recovered separately, according to the experimental protocol, and fixed in 70 % ethanol at -20 °C overnight. After washing cells twice with 1 mM PBS/EDTA, cells were recovered in PBS/EDTA

containing 20 $\mu\text{g/ml}$ RNase A at 37 °C for 1 h. Then, after one wash in PBS, cells were stained for DNA content for 30 min at 4 °C in the dark with 0.5 $\mu\text{g/ml}$ propidium iodide. Finally, cell cycle progression was analysed with FACScan, Beckman coulter Epics® XL cytofluorimeter. During FACS analysis, gating was carefully performed in order to exclude clumped cells and sub-diploid cell debris, and the relative percentage of cells in the different phases of the cell cycle was assessed as previously described [21].

PARP (PAR polymerase) and caspase activation

For Western blot analyses, total protein extracts (equivalent to 50 μg of protein/lane) were heated at 100 °C for 5 min in SDS/PAGE loading buffer (1:1, v/v) under reducing conditions and separated by electrophoresis under reducing conditions on 12 % (w/v) polyacrylamide gels. Then proteins were transferred to PVDF membranes as described above. PARP activation was determined using a mouse monoclonal anti-(human PARP) antibody (dilution, 1:1000) from Santa Cruz Biotechnology, (Santa Cruz, CA, U.S.A.). Caspases activation was studied by Western blot analysis as described above using polyclonal antibodies against human caspase-3, -8 and -9 (Santa Cruz Biotechnology) used at 1:1000 dilution. Four identical membranes loaded with the same samples for each experiment were produced and analysed for simultaneous determination of PARP, caspase-3, -8 and -9 in the same samples. Then each membrane was stripped three times and re-probed serially with all three different antibodies for better confirmation of the results.

Immunocytochemistry analysis

Controls, KSFM- or etoposide-treated cells (etoposide was from Sigma) were fixed with methanol/acetone (2:1) solution at 4 °C for 10 min, rinsed twice with PBS at 22 °C for 2 min, then re-hydrated with PBS plus 1 % (w/v) BSA for 10 min at 22 °C. Then cells were challenged with blocking solution [20 μl of normal goat serum in 5 ml of PBS plus 1 % (w/v) BSA] for 20 min and subjected to immunocytochemistry as described previously [21] using mouse monoclonal anti-(human clusterin) antibody (dilution, 1:50), clone 41D from Upstate Biotechnology. For detection of clusterin, secondary anti-mouse Alexa Fluor™ 568 red fluorescence (dilution 1:200) antibody from Molecular Probes (Leiden, The Netherlands) was used. Fluorescence microscopy analysis was performed with a fluorescence microscope, and digital images were acquired with a colour digital camera directly through the microscope as described above. Three independent immunocytochemistry experiments were performed for each experimental condition and three different fields were randomly chosen for data analysis.

Statistical analysis

Results are expressed as the means $\pm$ S.D. for the indicated number of independent determinations. The statistical significance was calculated by the Student's *t* test, and *P* values are indicated in the Figure legends and text.

RESULTS

Induction of anoikis in PNT1A cells upon KSFM incubation

Upon culturing in 10% serum-fed standard condition (RPMI), PNT1A cells growth was normal (Figure 1A) and cells showed a typical cobblestone morphology when confluence was reached at 8 days, as shown by Giemsa and DAPI nuclear staining

(Figures 1D and 1H respectively). In contrast, under serum-free conditions (KSFM), PNT1A cell morphology changed and cell growth was significantly decreased at 6 and 8 days of culture (Figure 1A; **P* < 0.05 and ***P* < 0.01 respectively, versus time-matched standard RPMI controls). Under these conditions, cell viability decreased (Figure 1B; **P* < 0.05 at 6 days and ***P* < 0.01 at 8 days, versus time-matched RPMI controls) as assessed by WST-1 cell viability assay. A progressive detachment of cells from wells (anoikis) and an increase in cell death was observed (Figure 1O; ***P* < 0.01 at 8 days versus time-matched standard RPMI control) on the basis of cell morphology (membrane and nuclear condensation, see the Experimental section for details).

Serum-free growth condition induced, first of all, rounding up and retraction of PNT1A cells, resulting in elongated rod shape (Figure 1E). Then, long-time KSFM exposure (Figure 1F) caused detachment of cells as single, individual elements (Figure 1F and 1L). Cell detachment, chromatin condensation and formation of apoptotic bodies, all indicators of anoikis induction [29], were also evident at this stage (Figure 1F and 1L, arrowheads). As seen in Figure 1, this phenomenon appears to be a slow process, in which cell death is high about 6 days after pro-apoptotic stimulation, as shown by apoptotic figures counting by DAPI nuclear staining (Figure 1O). Conversely, 8-day incubation in KSFM supplemented with FBS was effective at inhibiting PNT1A cells detachment. Under these conditions, cells reached a level of confluence very similar to that showed under standard culturing conditions (Figures 1M and 1N).

Cell detachment induces activation of caspases

When cells were grown in KSFM medium, an evident increase in caspase-3, -8 and -9 activation and PARP cleavage was detected after 8 days of culture (Figure 2A) by means of Western blot analysis. Caspase-3 activity was also determined in parallel, and the result obtained confirmed a significant increase in caspase-3 activity (Figure 2B) at 8 days of KSFM incubation (***P* < 0.01 versus time-matched standard RPMI control). A significant increase of caspase-3 activity was also determined after 8-day RPMI incubation, probably due to over-confluency of cells (#*P* < 0.05 versus 2-day standard RPMI control). Thus, specific cleavage of pro-caspase 3 (32 kDa) into the active caspase-3 fragments (17–10 kDa) and the cleavage of PARP (85 kDa) demonstrated that caspase-3-dependent apoptotic pathway was associated with cell detachment in PNT1A cells as a late event, significantly detectable only after 8 days of culture.

Accumulation of an intracellular 45 kDa clusterin isoform is associated with anoikis induction

On day 2 of culturing in RPMI, PNT1A cells synthesize clusterin as an intracellular precursor of about 65 kDa, which is apparently processed and converted into low-molecular-mass isoforms that are normally found in cell culture supernatants [41]. The production of these low-molecular-mass isoforms is generally dependent on cell density, and is usually higher when PNT1A cells reach confluence and proliferation rate is reduced. These protein products can also be found in the cell extracts and are usually secreted, under reducing conditions, as a triplet of bands showing, in our hands, an apparent size ranging from 40 to 45 kDa. Concomitantly, the secreted extracellular form of clusterin accumulates in the media (Figure 3A, cell extracts and cell culture media, RPMI). On the contrary, the same cells grown for up to 8 days in KSFM showed a significant decrease of the intracellular clusterin precursor, which was progressively converted into a 45 kDa intracellular isoform that was clearly less efficiently

Figure 1 Decreased cell growth and induction of cell detachment and anoikis death in PNT1A cells cultured in KFSM medium

(A) Cell growth of PNT1A cells under standard serum-fed (continuous line) or serum-free (broken line) conditions. Cell proliferation was assessed as described in the Experimental section. *D* values are expressed as the means $\pm$ S.D. from 3 independent experiments. Error bars indicating standard deviations are provided on each time point. Significant differences between serum-free and serum-fed conditions were: * $P < 0.05$ and ** $P < 0.01$. (B) Cell viability assay of PNT1A cells under standard serum-fed (○) or serum-free (△) conditions. At the indicated times, viability was assessed using the WST-1 cell viability assay as described in the Experimental section. Values are expressed as the means $\pm$ S.D. from 3 independent experiments. Significant differences between serum-free and serum-fed conditions were: * $P < 0.05$ and ** $P < 0.01$. (C–N) After incubation in standard RPMI (serum-fed), KFSM (serum-free) or KFSM + FBS conditions, cells were fixed and stained with Giemsa (C–F and M) or DAPI (Hoechst 33342) (G–L and N) for nuclear staining, as described in the Experimental section. Note that (C–F) and (M), compared with (G–L) and (N), show different fields, because Giemsa and DAPI staining cannot be performed in the same cell preparation. Cell rounding up starts at day 2 (E and I), well before the beginning of cell detachment. Detachment and accumulation of apoptotic bodies became evident at day 8 (F and L respectively, arrowheads). FBS supplementation completely rescued the cells (M and N). Magnification: (C–F) and (M), $\times 20$; (G–L) and (N), $\times 40$. Note the normal cobblestone morphology of cells upon RPMI incubation in (D), and chromatin condensation and apoptotic bodies formation in KFSM-grown cells (F and L). (O) Quantitative analysis of apoptosis by morphological changes. Cells were grown in standard RPMI (serum-fed), KFSM (serum-free) or KFSM + FBS condition. Then, DAPI nuclear staining was measured both in adherent and floating cells by directly adding the fluorescent dye to the cell culture medium. Positively stained cells were scored as normal or apoptotic on the basis of cellular morphology, as described in the Experimental section. Approx. 150 cells were counted in 3 different fields per condition and the percentage of apoptotic cells is shown. Values are expressed as the means $\pm$ S.D. obtained in 3 independent experiments. Significant differences between serum-free and serum-fed conditions were: ** $P < 0.01$.

secreted in the culture media (Figure 3A, cell extracts and cell culture media, KFSM).

PARP is catalytically activated by DNA ends or single-strand breaks and plays an auxiliary role in DNA repair processes and

apoptosis [42]. This enzyme catalyses the poly(ADP-ribosylation) of various nuclear proteins using NAD^+ as substrate during the early reversible stages of apoptosis [43,44]. Thus we studied PARP activation by proteolytic cleavage (Figure 3A, PARP)

Figure 2 Caspase-3 activity assay and activation of caspase-3, -8 and -9 and PARP during serum-free induced anoikis

(A) After seeding PNT1A cells under standard conditions (RPMI) for 24 h, cells were then switched to KFSM (serum-free) media and harvested at day 2 (lane 3) or day 8 (lane 4) of culture in comparison with time-matched RPMI controls (lanes 1 and 2 respectively). Equal amounts (50 μ g) of total protein cell extracts were loaded on to gels and resolved by SDS/PAGE, as described in the Experimental section. Western blot analysis of cytokeratins expression is provided to show equal loading of the samples. The picture shown is representative of 3 experiments performed independently, which gave the same results. (B) Caspase-3 activity in PNT1A cells grown under standard serum-fed (RPMI) or serum-free (KFSM) conditions. After seeding PNT1A cells with RPMI medium for 24 h, cells were then switched to KFSM medium and harvested at day 2 (bar 3) or day 8 (bar 4) culturing in comparison with time-matched controls kept in RPMI (bars 1 and 2). Then, caspase-3 activity was assessed by measuring fluorescence intensity of pNA, the proteolytic fragment of DEVD-pNA as described in the Experimental section. Values are expressed as the means $\pm$ S.D. from 3 independent experiments. Significant differences between KFSM and time-matched RPMI controls, or day 8 RPMI and day 2 RPMI, were: * $P < 0.05$ and ** $P < 0.01$, or # $P < 0.05$. Note that caspase-3, -8 and -9 and PARP are cleaved at 8 days of culturing under serum-free (KFSM) conditions (A) in association with the highest levels of caspase-3 activity (B).

and PARP activity, using antibodies directed against poly(ADP-ribose)ated nuclear proteins (Figure 3A, PAR), both by Western blot analysis. In fact, during apoptotic cell death, early and transient induction of PARP activity causes PAR accumulation

Figure 3 Changes in clusterin proteomic profile and mRNA expression in PNT1A cells following exposure to serum-fed (RPMI) or serum-free (KFSM) conditions

(A) Proteomic profile of clusterin. After seeding PNT1A cells under standard conditions (RPMI) for 24 h, cells were then switched to RPMI or KFSM media and harvested after 2- (lane a), 4- (lane b), 6- (lane c) or 8-day (lane d) culture in comparison with time-matched standard RPMI controls. Equal amounts of total cell extracts (50 μ g of protein) or culture medium (50 μ l) were collected in parallel, resolved by SDS/PAGE and transferred on to membranes for the indicated times (lanes a-d). Cytokeratins expression and Red Ponceau S staining of the blots are provided to show equal loading of the gels and transfer efficiency on to membranes. The gel shown is representative of 3 experiments performed independently, which gave the same results. Please note that cytokeratins are not detectable in the cell culture medium. Changes in the proteomic profile of clusterin, leading to the accumulation of a 45 kDa clusterin isoform at day 8 of KFSM culturing, are evident following exposure of cells to KFSM. Under the same conditions, the amount of clusterin secreted in the cell culture medium is strongly decreased when compared with RPMI growth condition. Progressive PARP cleavage and decreased poly(ADP-ribose)ation in KFSM samples demonstrate that apoptosis activation was massive at day 8 (d). (B) Northern blot analysis of clusterin and histone H3 (specific marker of the S-phase) mRNAs in total RNA preparations from PNT1A cells grown under serum-fed (RPMI) or serum-free (KFSM) conditions for the indicated times (lanes a and d, see above). The picture shown is representative of 3 experiments performed independently, which gave the same results. The level of the mRNA coding for GAPDH, a typical house-keeping gene, and ethidium bromide staining of the gel were also shown for comparison. The initial decrease in clusterin mRNA at day 2 (KFSM, lane a) is accompanied by a strong decrease in histone H3 mRNA, that continues up to day 8 (KFSM, lane d) of culturing, suggesting that inhibition of clusterin gene transcription and post-translational conversion of the constitutively accumulated 65 kDa precursor to 45 kDa clusterin isoform are associated with early decrease of cell proliferation.

in early apoptotic cells [42] by transferring ADP-ribose moieties to nuclear acceptor proteins and to the enzyme itself [44]. For this reason, the detection of PAR is considered an early marker

of apoptosis. In fact, poly(ADP-ribosyl)ation of nuclear proteins was detectable as early as 2 days after starting serum-free growth condition (Figure 3A), well before the detection of caspase-3 activity (Figure 2B). At this early stage of anoikis induction (2-day), cells were still viable (as assessed by Trypan Blue exclusion test, results not shown) and can be rescued by re-plating them with standard serum-fed RPMI medium (results not shown). The early burst of poly(ADP-ribosyl)ation of nuclear proteins at day 2 of KSFM incubation was then followed by a marked decline in poly(ADP-ribosyl)ation at day 8 of treatment, when chromatin condensation and formation of apoptotic bodies were already massive (Figures 1F and 1L). Thus PARP activation and decreased PAR at 8 days of culture in KSFM demonstrated that apoptosis effectors activation was a rather late event, elicited when changes in clusterin proteomic profile were already maximal. In fact, changes in clusterin proteomic profile and accumulation of the 45 kDa intracellular clusterin isoform preceded high levels of cell-growth inhibition (Figure 1A), formation of apoptotic bodies (Figures 1F and 1L) and cell detachment, and were associated with PARP activation.

Interestingly, the accumulation of the 45 kDa isoform was concomitant to a decrease in the accumulation of clusterin mRNA (Figure 3B), strongly suggesting that this event might be produced mainly by post-translational events. At the same time, histone H3 mRNA level, which is a specific molecular marker of the S-phase of the cell cycle (Figure 3B), was clearly lower in KSFM-treated cells, consistently with cell growth inhibition conditions. GAPDH (an housekeeping gene) mRNA level is shown for comparison and shows no major changes. The initial decrease in clusterin mRNA at day 2, accompanied by a strong decrease in histone H3 mRNA up to the 8th day of culture, clearly shows that inhibition of clusterin gene transcription and post-translational conversion of the constitutively accumulated 65 kDa precursor into 45 kDa clusterin isoform are events closely associated with decreased cell proliferation under KSFM conditions.

The 45 kDa clusterin isoform appears to be specifically produced by post-translational modification of the protein precursor that is constitutively expressed. The fact that 45 kDa clusterin isoform could be produced by caspases activity can be excluded. In fact, an analysis performed using the Peptidecutter software available at the ExPASy database (<http://cn.expasy.org/tools/peptidecutter/>) showed that caspase-1, -2, -3, -4, -6, -7, -8, -9 and -10 do not cleave clusterin at any site.

FAK dephosphorylation and down-regulation of poly(ADP-ribosyl)ation are concomitant with cell detachment

To understand better the timing of the events leading to cell detachment and apoptosis, we studied several biochemical parameters from day 2 to 8 of culturing in two different cell populations recovered separately: (i) the adhering cells which survived exposure to KSFM (Figure 4, adhering cells), and (ii) the floating cells (responsive to KSFM treatment) which were detached from the plates. These cells were collected separately by gentle tapping of the plates and centrifugation (Figure 4, non-adhering cells). FAK was fully phosphorylated in adhering PNT1A cells under RPMI growth conditions, while a slight decrease in FAK phosphorylation was detected upon KSFM incubation. FAK expression did not change significantly at any time. FAK phosphorylation and expression were instead strongly decreased in non-adhering cells. In fact, both FAK and p-FAK-Tyr³⁹⁷ were clearly down-regulated in non-adhering cells, starting at day 2 and up to day 8 of KSFM culturing, when these cells were finally and irreversibly committed to anoikis (Figure 4, non-adhering cells).

Figure 4 45 kDa clusterin isoform specifically segregates in detached cells and apoptotic bodies originating from anoikis-committed cells

After seeding PNT1A cells under standard conditions for 24 h, the cells were switched to KSFM media for 2 (lane a), 6 (lane c) and 8 (lane d) days. Then, two different cell populations were collected separately: the adhering cells, which survived exposure to KSFM, and the non-adhering cells, responsive to KSFM, which detached from the plates. Cells were collected by gentle tapping of the plates and centrifugation. In parallel, adhering cells cultured in standard RPMI conditions for 2 days (lane a) were used for comparison. Equal amounts (50 μ g) of total cell protein extracts were loaded, resolved on the gel and transferred on to the membranes for each time point. The gel shown is representative of 3 experiments performed independently. Cytokeratins expression and Red Ponceau S staining of the blots are also provided to show equal loading of the samples. Changes in clusterin protein expression profile were strictly associated with significant down-regulation of p-FAK-Tyr³⁹⁷, FAK and PAR, and massive cleavage of PARP and cytocheratins in detached cells. Note that the 45 kDa clusterin isoform specifically and strongly accumulated in non-adhering cells from 2- (lane a) to 8-day (lane d) exposure to KSFM incubation when compared with cells not responsive and surviving to KSFM exposure (adhering cells).

Commitment to apoptosis is demonstrated by progressive PARP activation and PAR decrease in this cell population. Under these experimental conditions, FAK was not cleaved as previously described in floating human neuro-epithelioma cells undergoing ceramide-induced detachment and apoptosis [45]. Dephosphorylation of FAK and loss of focal contacts in PNT1A non-adhering cells were associated with complete inhibition of poly(ADP-ribosyl)ation (Figure 4, non-adhering cells), again indicating that, at days 6–8 of KSFM culturing, floating cells were already entering in the irreversible stage of apoptosis.

Taken together, the results presented in Figures 1, 2 and 4 show that KSFM exposure induces cell detachment before caspase cascade activation. This feature is typical of anoikis, in which adherent cells undergo apoptosis after disruption of epithelial

cell-matrix interaction [27]. In anoikis-committed floating cells the accumulation of the 45 kDa isoform of clusterin was dramatic, showing that segregation of this clusterin isoform, starting on the second day of KSFM culturing, efficiently continued in detached cells and apoptotic bodies, increasing dramatically up to day 8 of incubation (Figure 4, non-adhering cells). In the non-adhering cells, caspase-3 activation was associated with the cleavage of intermediate filament proteins, such as cytokeratins, as previously shown to occur for keratin-18 and -19 during apoptosis [46], but this did not cause degradation of the 45 kDa clusterin, which seemed to be rather stable, even when segregated in apoptotic bodies. As a matter of fact, this was predicted by the Peptidecutter software (<http://cn.expasy.org/tools/peptidecutter/>), because 45 kDa clusterin is not a substrate of known caspases.

Early nuclear translocation of the 45 kDa clusterin isoform inhibits cell cycle progression and primes cells for anoikis

Recent studies have described alterations in the biogenesis and intracellular trafficking of clusterin in MCF-7 cells undergoing apoptosis induced by anti-oestrogens and tumour necrosis factor α [47]. O'Sullivan et al. [47] showed the appearance of a 50–53 kDa uncleaved, non-glycosylated, disulphide-linked isoform of clusterin that accumulated in the nucleus of dying cells. Consistent with the results of O'Sullivan et al. [47], in PNT1A cells anoikis induction was preceded by early translocation of the 45 kDa clusterin isoform to the nucleus (Figure 5A, KSFM, nuclear extracts). In fact, nuclear translocation of the 45 kDa clusterin isoform was already detectable 2 days after serum-free growth well before commitment to anoikis and massive DNA fragmentation (Figures 1E and 1I), as assessed by FACS (Figure 5B, panel 3). Clusterin (45 kDa) progressively accumulated in the nuclei up to day 8, in strict association with the disappearance of the 45 kDa cytoplasm form of clusterin and precursor (Figure 5A, KSFM and RPMI respectively, cytoplasm extracts). Noticeably, nuclear clusterin was undetectable in control cells at any time. At day 2 of KSFM culturing, cell cycle progression was already affected, particularly in G₂/M phases (Figure 5B, panel 3; $^{**}P < 0.01$) as compared with controls (Figure 5B, panel 1). Under this condition, cells were primed for anoikis, but still not committed to die, since they can be rescued by switching them to standard growth conditions (RPMI + FBS; results not shown).

Clusterin translocation and accumulation into the nucleus appeared to be specifically associated with cell cycle arrest, similarly to what we have previously found by transient over-expression of clusterin in the same cells [21]. In absence of rescuing factors, long-time exposure to this condition and, particularly, long-term cell cycle arrest, would then irreversibly commit cells to death. In fact, at the late stage (day 8) of culturing in KSFM, accumulation of the 45 kDa clusterin isoform into the nucleus caused a massive accumulation of subdiploid cells (Figure 5B, panel 4, subdiploid peak, arrow; $^{**}P < 0.01$) and strong perturbation of cell cycle progression. Separate collection of adhering and non-adhering cells followed by FACS analysis showed a specific detachment of cells arrested at the G₀/G₁-S transition (Figure 5B, panel 6, non-adhering cells; $^{##}P < 0.01$). At the same time, surviving cells did not show major changes in cell cycle progression (Figure 5B, panel 5, adhering cells) as compared with 8-day culturing under serum-fed conditions (Figure 5B, panel 2), suggesting that is unlikely that clusterin might act as a survival gene, at least in this experimental model.

To confirm nuclear translocation of clusterin, we also performed an immunocytochemistry analysis of PNT1A cells for detection of intracellular trafficking of this protein under standard (RPMI) and serum-free (KSFM) cell growth conditions. As shown in

Figure 6, under RPMI-growth conditions clusterin was constantly localized to the cytoplasm (Figures 6A and 6B), but, as early as at day 2, translocation of clusterin to the nucleus was detected under KSFM-growth conditions (Figure 6C), just before the detection of major morphological changes in the cells (see Figures 1E and 1I) and the commitment to anoikis-death (see Figure 5B, panel 3). Later, at day 8 of KSFM culturing, nuclear clusterin localization was massive (Figure 6D) and the protein also accumulated into floating apoptotic bodies (results not shown).

To check whether nuclear localization of clusterin was only specifically caused by KSFM treatment of PNT1A cells, or else a common feature of other models of apoptosis, we also treated PNT1A cells with topoisomerase II inhibitor etoposide. Etoposide is a well-known chemotherapeutic drug capable of inducing apoptosis by enhancing enzyme-mediated DNA breaking in several cell lines [48]. As a matter of fact, the ability of etoposide to induce cell cycle arrest followed by apoptosis is well known in many biological models, including prostate cells [49]. Thus PNT1A cells treated with etoposide were subjected to immunocytochemistry and FACS analysis. As expected, 24 h of 50 μ M etoposide treatment were sufficient to induce a massive accumulation of subdiploid cells (Figure 6G, panel 2, subdiploid peak, arrow; $^{**}P < 0.01$ versus 24 h RPMI, panel 1) and a strong perturbation of cell cycle progression. Under these conditions, nuclear translocation of clusterin (Figure 6E) was observed in strict association with a significant increase in caspase-3 activity (Figure 6F; $^{**}P < 0.01$).

Thus clusterin translocation and accumulation into the nucleus is a common event associated with cell death induction, and appeared to be in strict relation to early cell cycle arrest at the G₀/G₁-S-phase checkpoint. This result is consistent with our previous findings obtained when clusterin was transiently over-expressed in the same cells [21], leading to the possible conclusion that nuclear clusterin might have anti-proliferative properties.

DISCUSSION

Tight control of cell proliferation is needed to ensure normal tissue steady-state growth and prevent cancer onset. Normal cells require adhesion to extracellular matrix for survival, and inadequate or inappropriate cell-matrix interaction can be responsible for cell-detachment-induced apoptosis, also known as anoikis [28]. In fact, changes in cell shape and cytoskeleton might be considered a potential cause of anoikis [32]. This peculiar cell death mechanism is critical for maintaining homeostasis and architecture of different organs and tissues, and epithelia in particular. Moreover, adhesion to the substrate affects cell growth, and the loss of anchorage-dependent growth is a hallmark of cell transformation [32]. In fact, tumour cell survival and spreading to inappropriate environments can promote metastasis formation [28]. Clusterin was previously found as one of the few genes specifically down-regulated by anchorage [35]. This gene has been suggested to produce several isoforms of clusterin protein, probably from a single mRNA, because of alternative post-translational modification [11,13,14]. These alternative isoforms could be involved either in the processes of cell death [10] or cell survival [9,23,50]. We report here a tight link between the induction of a 45 kDa nuclear clusterin isoform and anoikis induction in PNT1A cells grown under serum-free conditions.

For the first time to our knowledge, we have demonstrated that the native clusterin proteomic expression profile can change, at least in PNT1A cells, as a consequence of cell growth condition (Figure 3). The conversion of the constitutively expressed protein precursor to smaller products is an event that is strictly associated

Figure 5 45 kDa clusterin nuclear accumulation is associated with detachment of cells that are specifically blocked at the G₁–S boundary of the cell cycle

(A) Cells were seeded under standard conditions (RPMI) for 24 h, then switched to KSFM conditions and harvested at 2- (lane a), 4- (lane b), 6- (lane c) or 8-day (lane d) culture in parallel with time-matched RPMI controls used for comparison. Equal amounts (50 µg) of cell cytoplasm and nuclear extracts were prepared, resolved by SDS/PAGE and transferred on to membranes as described in the Experimental section. The gel shown is representative of 3 experiments performed independently. 45 kDa clusterin accumulation into the nucleus was already detectable at 2 days (lane a) of KSFM incubation and progressively increased up to 8 days (lane d), while the same protein form decreased in the cytoplasm extract. The nuclear form of clusterin was not detectable in the RPMI samples at any time, whereas cytoplasm extracts clearly show the presence of clusterin precursor and clusterin low-molecular-mass bands (ranging 40–45 kDa) under RPMI conditions. Consistently, these protein isoforms are not detectable in the corresponding nuclear extracts. Finally, the presence of cytokeratins only in the cytoplasm fraction demonstrated no major contamination of nuclear fraction by cytoplasm products. (B) Cell cycle progression analysis in total cell population cultured in RPMI (panels 1 and 2) or KSFM (panels 3 and 4) medium. The same analysis was also conducted separately on adhering (panel 5) and non-adhering (panel 6) cells. Subdiploid cells, and the relative percentage of cells in the different phases of the cell cycle, are also shown. Values are mean of cell population (percentage of total) ± S.D. obtained in five independent experiments. Significant differences were: ♦♦, 2 days KSFM (panel 3) versus 2 days RPMI (panel 1) ($P < 0.01$); §§, 8 days KSFM adhering cells (panel 5) versus 8 days RPMI (panel 2) ($P < 0.01$); ##, 8 days KSFM non-adhering cells (panel 6) versus 8 days RPMI (panel 2) ($P < 0.01$); **, 8 days KSFM (panel 4) versus 8 days RPMI (panel 2) ($P < 0.01$). Anoikis induction by KSFM exposure caused a massive increase in the subdiploid peak at 8 days (panel 4, total cell population, arrow, ** $P < 0.01$) and selective loss of a cell population that appears to be specifically blocked in the G₀/G₁–S transition (panel 6, non-adhering cells; ## $P < 0.01$), whereas cells surviving KSFM exposure did not show major changes when compared with controls (8 days RPMI).

with suppression of cell proliferation (Figures 1A and 1B) and apoptosis induction (Figures 1O, 2 and 4). In particular, the formation and translocation of an alternative clusterin isoform

into the nucleus (Figures 5A and 6A–6D) was an early specific response that preceded the morphological hallmarks of apoptosis (Figures 1F and 1L, arrows), leading to cell cycle arrest at the

Figure 6 Nuclear targeting of clusterin is an early event upon KSFM incubation and etoposide administration

(A–D) PNT1A cells were grown under standard RPMI or KSFM conditions for 2 and 8 days, then fixed and used for immunocytochemistry analysis as described in the Experimental section. Under RPMI conditions, clusterin was detected in the cytoplasm (A and B). Under KSFM conditions, clusterin was already detected into the nuclei of adhering cells (C) at day 2 of culturing, whereas later clusterin nuclear localization was massive (D) and its accumulation was also detectable in floating apoptotic bodies (results not shown). Magnification, $\times 20$. (E) PNT1A cells were given 50 μ M etoposide for 24 h in RPMI medium, then fixed and used for immunocytochemistry analysis as described in the Experimental section. Note that nuclear translocation of clusterin was already detected 24 h after etoposide administration. Magnification, $\times 20$. (F) 50 μ M etoposide was added to PNT1A cells for 24 h in RPMI medium. Then caspase-3 activity was assessed as indicated in the legend to Figure 2. Values are expressed as the means $\pm$ S.D. from 3 independent experiments. Significant differences between RPMI + etoposide and RPMI control were $**P < 0.01$. (G) Cell cycle progression analysis in total cell population cultured in RPMI (panel 1) or RPMI + etoposide (panel 2) medium for 24 h. Subdiploid cells, and the relative percentage of cells in the different phases of the cell cycle, are also shown. Values are mean of cell population (percentage of total) $\pm$ S.D. from 3 independent experiments. Significant differences are $*P < 0.05$ and $**P < 0.01$, 24 h incubation with RPMI + etoposide (panel 2) versus 24 h incubation with RPMI (panel 1).

G₀/G₁–S boundary (Figure 5B), and thus priming cells to anoikis (Figures 2 and 4). Nuclear targeting of this specific 45 kDa isoform of clusterin, that is likely due to post-translational modification,

since clusterin mRNA accumulation decreased under the same experimental conditions (Figure 3B), appears to be an early molecular signal activated by environmental/hormonal stress, being already detectable at day 2 of serum-free culturing (Figures 5A and 6A–6D). We suggest here that this event would be responsible for causing the slowing down of cell cycle progression, extending the time interval between G₁ and G₂/M phases, finally arresting cells in the G₁ and S-phase (Figure 5B, panel 5). This would lead to selective detachment of cells which are blocked at the G₁/S transition (Figure 5B, compare panels 5 and 6; $**P < 0.01$). The loss of anchorage-dependent growth would prime, but not commit, cells to anoikis death (Figure 5B, panel 6; $**P < 0.01$). In fact, early (within 24 h following KSFM exposure) FBS supplementation to KSFM was effective at rescuing early-detached PNT1A cells (Figures 1M and 1N, and results not shown). This is consistent with the fact that it has been shown that cell detachment, caused by suppression of integrins activity or disruption of the integrin/extracellular matrix survival signals and growth factor receptors network [27,51], are events being well upstream of activation of apoptosis effectors genes, such as caspases [51]. On the other hand, long-time exposure to the same conditions, in absence of rescuing factors, would commit cells to definitive apoptotic death, probably because of long-term extension of cell cycle arrest at the G₁/S transition checkpoint. We also reported here that early clusterin localization to the nucleus, in immortalized human prostate epithelial cells, may be a common event following other apoptosis stimuli, such as etoposide administration, suggesting that early clusterin nuclear targeting (Figure 6E) is a key event in strict association with induction of apoptosis (Figure 6G, panel 2) and increase in caspase-3 activity (Figure 6F).

The 45 kDa clusterin isoform appears to be retained in the cell (Figure 3A, total cell extracts) and not secreted (Figure 3A, cell culture medium), accumulating in floating cells and apoptotic bodies (Figure 4). These experimental results are consistent with the recent finding that clusterin expression was shown to be repressed by anchorage using DNA microarray analysis [35]. The fact that, upon serum starvation, clusterin accumulation was high only in non-adhering cells was demonstrated by concomitant dephosphorylation of FAK in these cells (Figure 4). Concomitantly to clusterin accumulation, PARP activation was also high in non-adhering cells at late (8 day) anoikis induction, in association with complete inhibition of poly(ADP-ribosylation) (Figure 4). In fact, at this late stage of anoikis, the stable PAR moieties bound to nuclear proteins were degraded by cleavage, showing that cell death has already entered an irreversible stage along the apoptotic pathway. During apoptosis, it is well known that the activity of caspases is responsible for the inactivation of the poly(ADP-ribosylation) process [44]. Consistently, under these conditions, cells showed apoptosis-associated proteolysis of cytokeratins (Figure 4, non-adhering cells). Cytokeratins are known to be degraded during late stages of apoptosis [46].

The novelty of our study is that it is first experimental observation that suppression of cell growth, decreased DNA synthesis and cell cycle arrest at the G₀/G₁–S boundary were closely associated with early progressive translocation to the nucleus of a native 45 kDa alternative isoform of clusterin, also confirming our previous study [21] and observations by others [12,52,53]. It must be stressed that the results presented here, to our knowledge, are the first experimental evidences that nuclear clusterin can be detected in prostate epithelial cells as a native protein under a pro-apoptotic stimulus and not as a GFP (green fluorescent protein)-fusion product following over-expression in a MCF-7 cell transfection system [12]. In any case, both results are consistent with the hypothesis that clusterin has an important

role in the regulation of cell proliferation. These results agree well with our previous findings that clusterin is down-regulated during prostate cancer onset and progression [17,54]. This observation was recently fully confirmed by the pattern of expression of clusterin determined in 62 primary prostate tumours compared with 41 normal prostate specimens and 9 lymph node metastases [55] (please search for 'clusterin' in the online database at <http://microarray-pubs.stanford.edu/cgi-bin/gx?n=prostate1&rx=5>), leading to the conclusion that a clusterin isoform, acting as putative anti-proliferative and potentially pro-apoptotic intracellular signal, could play a peculiar role in preventing pathological proliferation of prostate epithelial cells and cancer growth, and suggesting that clusterin could be a new tumour-suppressor gene in the prostate [21]. At least in the PNT1A cells model, clusterin nuclear translocation would cause cell cycle arrest primarily, priming prostate epithelial cells to slow-death (i.e. anoikis) and caspase-3 activation. The fact that caspase-3 independent cell death was suggested in other experimental systems [12,56] seems not in contradiction with our findings, since inhibition of cell proliferation caused by nuclear clusterin accumulation might very likely activate (although indirectly) different apoptotic pathways according to the cell metabolic contest.

Our results also suggest a possible explanation for apparently conflicting results reported in the scientific literature. In fact, we hypothesize that changes in the steady-state balance between different isoforms of clusterin could differently affect cell growth, possibly priming cells to death or allowing cell survival instead. This would account for the many reports suggesting totally different functions played by this protein in different biological systems. In this view, alternative isoforms of clusterin could participate in processes that may have opposite effects on the cell. In addition, cells might tolerate clusterin over-expression (eventually induced by pro-apoptotic signals) if rescuing factors can balance clusterin action or if such cells have acquired a phenotype that is resistant to apoptotic cell death. It must be also considered that viable cells over-expressing clusterin can be transiently detected for a short time, at least in our experimental system, since clusterin seems to prime cells to a slow-acting death program, but they would eventually die later on if not rescued. Finally, the lack of clusterin accumulation in adhering cells surviving for 8 days to KSFM growth conditions do not support the hypothesis that clusterin might confer survival advantages, thus being an anti-apoptotic gene.

The development of new antibodies capable of specifically detecting different clusterin isoforms, possibly produced under standard growth conditions or following apoptotic stimuli, could enable us to isolate and study the structurally distinct clusterin protein family members that appear to be produced from the precursor, challenging the hypothesis that different forms of this protein could play distinct functions in normal and pathological prostate. This work is currently in progress in our laboratory. The detection of specific human clusterin isoforms linked to cell death could become a new molecular tool for the characterization of prostate cancer progression.

This work was partially supported by AIRC 2002-2003, Investigator Grant, title 'Clusterin and polyamine regulatory genes as new molecular markers for prostate cancer prognosis and therapy' and Ministero dell'Istruzione, dell'Università e della Ricerca (Fondo Integrativo Speciale per la Ricerca 2001).

REFERENCES

- Rosenberg, M. E. and Silksens, J. (1995) Clusterin: physiologic and pathophysiological considerations. *Int. J. Biochem. Cell Biol.* **27**, 633–645
- Purrello, M., Bettuzzi, S., Di Pietro, C., Mirabile, E., Di Blasi, M., Rimini, R., Grzeschik, K. H., Ingletti, C., Corti, A. and Sichel, G. (1991) The gene for SP-40,40, human homolog of rat sulfated glycoprotein 2, rat clusterin, and rat testosterone-repressed prostate message 2, maps to chromosome 8. *Genomics* **10**, 151–156
- Fink, T. M., Zimmer, M., Tschopp, J., Etienne, J., Jenne, D. E. and Lichter, P. (1993) Human clusterin (CL) maps to 8p21 in proximity to the lipoprotein lipase (LPL) gene. *Genomics* **16**, 526–528
- Murphy, B. F., Kirsbaum, L., Walker, I. D. and d'Apice, A. J. (1988) SP-40,40, a newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. *J. Clin. Invest.* **81**, 1858–1864
- Bettuzzi, S., Hiipakka, R. A., Gilna, P. and Liao, S. T. (1989) Identification of an androgen-repressed mRNA in rat ventral prostate as coding for sulphated glycoprotein 2 by cDNA cloning and sequence analysis. *Biochem. J.* **257**, 293–296
- Jones, S. E. and Jomary, C. (2002) Clusterin. *Int. J. Biochem. Cell Biol.* **34**, 427–431
- Poon, S., Treweek, T. M., Wilson, M. R., Easterbrook-Smith, S. B. and Carver, J. A. (2002) Clusterin is an extracellular chaperone that specifically interacts with slowly aggregating proteins on their off-folding pathway. *FEBS Lett.* **513**, 259–266
- Humphreys, D. T., Carver, J. A., Easterbrook-Smith, S. B. and Wilson, M. R. (1999) Clusterin has chaperone-like activity similar to that of small heat shock proteins. *J. Biol. Chem.* **274**, 6875–6881
- Koch-Brandt, C. and Morgans, C. (1996) Clusterin: a role in cell survival in the face of apoptosis? *Prog. Mol. Subcell. Biol.* **16**, 130–149
- Yang, C. R., Yeh, S., Leskov, K., Odgaard, E., Hsu, H. L., Chang, C., Kinsella, T. J., Chen, D. J. and Boothman, D. A. (1999) Isolation of Ku70-binding proteins (KUBs). *Nucleic Acids Res.* **27**, 2165–2174
- Yang, C. R., Leskov, K., Hosley-Eberlein, K., Criswell, T., Pink, J. J., Kinsella, T. J. and Boothman, D. A. (2000) Nuclear clusterin/XIP8, an X-ray-induced Ku70-binding protein that signals cell death. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5907–5912
- Leskov, K. S., Klovov, D. Y., Li, J., Kinsella, T. J. and Boothman, D. A. (2003) Synthesis and functional analyses of nuclear clusterin, a cell death protein. *J. Biol. Chem.* **278**, 11590–11600
- Kimura, K. and Yamamoto, M. (1996) Modification of the alternative splicing process of testosterone-repressed prostate message-2 (TRPM-2) gene by protein synthesis inhibitors and heat shock treatment. *Biochim. Biophys. Acta* **1307**, 83–88
- Lakins, J., Bennett, S. A., Chen, J. H., Arnold, J. M., Morrissey, C., Wong, P., O'Sullivan, J. and Tenniswood, M. (1998) Clusterin biogenesis is altered during apoptosis in the regressing rat ventral prostate. *J. Biol. Chem.* **273**, 27887–27895
- Bettuzzi, S., Strocchi, P., Marinelli, M., Astancolle, S., Davalli, P. and Corti, A. (1994) Gene relaxation and aging: changes in the abundance of rat ventral prostate SGP-2 (clusterin) and ornithine decarboxylase mRNAs. *FEBS Lett.* **348**, 255–258
- Bettuzzi, S., Astancolle, S., Guidetti, G., Moretti, M., Tiozzo, R. and Corti, A. (1999) Clusterin (SGP-2) gene expression is cell cycle dependent in normal human dermal fibroblasts. *FEBS Lett.* **448**, 297–300
- Bettuzzi, S., Davalli, P., Astancolle, S., Carani, C., Madeo, B., Tampieri, A. and Corti, A. (2000) Tumor progression is accompanied by significant changes in the levels of expression of polyamine metabolism regulatory genes and clusterin (sulfated glycoprotein 2) in human prostate cancer specimens. *Cancer Res.* **60**, 28–34
- Petropoulos, C., Trougakos, I. P., Kolettas, E., Toussaint, O. and Gonos, E. S. (2001) Clusterin/apolipoprotein J is a novel biomarker of cellular senescence that does not affect the proliferative capacity of human diploid fibroblasts. *FEBS Lett.* **509**, 287–297
- Astancolle, S., Guidetti, G., Pinna, C., Corti, A. and Bettuzzi, S. (2000) Increased levels of clusterin (SGP-2) mRNA and protein accompany rat ventral prostate involution following finasteride treatment. *J. Endocrinol.* **167**, 197–204
- Crescioli, C., Ferruzzi, P., Caporali, A., Mancina, R., Comerchi, A., Muratori, M., Scaltriti, M., Vannelli, G. B., Smirardo, S., Mariani, R. et al. (2003) Inhibition of spontaneous and androgen-induced prostate growth by a nonhypercalcemic calcitriol analog. *Endocrinology* **144**, 3046–3057
- Bettuzzi, S., Scorcioni, F., Astancolle, S., Davalli, P., Scaltriti, M. and Corti, A. (2002) Clusterin (SGP-2) transient overexpression decreases proliferation rate of SV40-immortalized human prostate epithelial cells by slowing down cell cycle progression. *Oncogene* **21**, 4328–4334
- Miyake, H., Nelson, C., Rennie, P. S. and Gleave, M. E. (2001) Acquisition of chemoresistant phenotype by overexpression of the antiapoptotic gene testosterone-repressed prostate message-2 in prostate cancer xenograft models. *Cancer Res.* **60**, 2547–2554
- Miyake, H., Gleave, M. E., Arakawa, S., Kamidono, S. and Hara, I. (2002) Introducing the clusterin gene into human renal cell carcinoma cells enhances their metastatic potential. *J. Urol.* **167**, 2203–2208
- Bettuzzi, S., Troiano, L., Davalli, P., Tropea, F., Ingletti, M. C., Grassilli, E., Monti, D., Corti, A. and Franceschi, C. (1991) *In vivo* accumulation of sulfated glycoprotein 2 mRNA in rat thymocytes upon dexamethasone-induced cell death. *Biochem. Biophys. Res. Commun.* **175**, 810–815

- 25 Leskov, K. S., Criswell, T., Antonio, S., Li, J., Yang, C. R., Kinsella, T. J. and Boothman, D. A. (2001) When X-ray-inducible proteins meet DNA double strand break repair. *Semin. Radiat. Oncol.* **11**, 352–372
- 26 Meredith, Jr, J. E., Fazeli, B. and Schwartz, M. A. (1993) The extracellular matrix as a cell survival factor. *Mol. Biol. Cell* **4**, 953–961
- 27 Frisch, S. M. and Francis, H. (1994) Disruption of epithelial cell–matrix interactions induces apoptosis. *J. Cell Biol.* **124**, 619–626
- 28 Frisch, S. M. and Sreaton, R. A. (2001) Anoikis mechanisms. *Curr. Opin. Cell Biol.* **13**, 555–562
- 29 Jiang, C., Wang, Z., Ganther, H. and Lu, J. (2001) Caspases as key executors of methyl selenium-induced apoptosis (anoikis) of DU-145 prostate cancer cells. *Cancer Res.* **61**, 3062–3070
- 30 Gilmore, A. P., Metcalfe, A. D., Romer, L. H. and Streuli, C. H. (2000) Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization. *J. Cell Biol.* **149**, 431–446
- 31 Kiechle, F. L. and Zhang, X. (2002) Apoptosis: biochemical aspects and clinical implications. *Clin. Chim. Acta* **326**, 327–345
- 32 Huang, S. and Ingber, D.-E. (1999) The structural and mechanical complexity of cell-growth control. *Nat. Cell Biol.* **1**, 131–138
- 33 van de Water, B., Houtepen, F., Huigsloot, M. and Tjeldens, I. B. (2001) Suppression of chemically induced apoptosis but not necrosis of renal proximal tubular epithelial (LLC-PK1) cells by focal adhesion kinase (FAK). Role of FAK in maintaining focal adhesion organization after acute renal cell injury. *J. Biol. Chem.* **276**, 36183–36193
- 34 Lee, E. C., Zhan, P., Schallhorn, R., Packman, K. and Tenniswood, M. (2003) Antiandrogen-induced cell death in LNCaP human prostate cancer cells. *Cell Death Differ.* **10**, 761–771
- 35 Goldberg, G. S., Jin, Z., Ichikawa, H., Naito, A., Ohki, M., El Deiry, W. S. and Tsuda, H. (2001) Global effects of anchorage on gene expression during mammary carcinoma cell growth reveal role of tumor necrosis factor-related apoptosis-inducing ligand in anoikis. *Cancer Res.* **61**, 1334–1337
- 36 Cussenot, O., Berthon, P., Berger, R., Mowszowicz, I., Faille, A., Hojman, F., Teillac, P., Le Duc, A. and Calvo, F. (1991) immortalization of human adult normal prostatic epithelial cells by liposomes containing large T-SV40 gene. *J. Urol.* **146**, 881–886
- 37 Blanchere, M., Berthaut, I., Portois, M. C., Mestayer, C. and Mowszowicz, I. (1998) Hormonal regulation of the androgen receptor expression in human prostatic cells in culture. *J. Steroid Biochem. Mol. Biol.* **66**, 319–326
- 38 Chaproniere, D. M. and McKeenan, W. L. (1986) Serial culture of single adult human prostatic epithelial cells in serum-free medium containing low calcium and a new growth factor from bovine brain. *Cancer Res.* **46**, 819–824
- 39 Berthon, P., Waller, A. S., Villette, J. M., Lorodin, L., Cussenot, O. and Maitland, N. J. (1997) Androgens are not a direct requirement for the proliferation of human prostate epithelium *in vitro*. *Int. J. Cancer* **73**, 910–916
- 40 Dooley, T. P., Gadwood, R. C., Kilgore, K. and Thomasco, L. M. (1994) Development of an *in vitro* primary screen for skin depigmentation and antimelanoma agents. *Skin Pharmacol.* **7**, 188–200
- 41 Humphreys, D., Hochgrebe, T. T., Easterbrook-Smith, S. B., Tenniswood, M. P. and Wilson, M. R. (1997) Effects of clusterin overexpression on TNF α - and TGF β -mediated death of L929 cells. *Biochemistry* **36**, 15233–15243
- 42 Rosenthal, D. S., Ding, R., Simbulan-Rosenthal, C. M., Vaillancourt, J. P., Nicholson, D. W. and Smulson, M. E. (1997) Intact cell: Evidence for the early synthesis, and subsequent late apoptotic-mediated suppression, of poly(ADP-ribose) during apoptosis. *Exp. Cell Res.* **232**, 313–321
- 43 Simbulan-Rosenthal, C. M., Rosenthal, D. S., Iyer, S., Boulares, H. and Smulson, M. E. (1999) Involvement of PARP and poly(ADP-ribosylation) in the early stages of apoptosis and DNA replication. *Mol. Cell. Biochem.* **193**, 137–148
- 44 Soldani, C. and Scovassi, A. I. (2002) Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: An update. *Apoptosis* **7**, 321–328
- 45 Di Bartolomeo, S. and Spinedi, A. (2002) Ordering ceramide-induced cell detachment and apoptosis in human neuroepithelioma. *Neurosci. Lett.* **334**, 149–152
- 46 Ku, N. O. and Omary, M. B. (2001) Effect of mutation and phosphorylation of type I keratins on their caspase-mediated degradation. *J. Biol. Chem.* **276**, 26792–26798
- 47 O'Sullivan, J., Whyte, L., Drake, J. and Tenniswood, M. (2003) Alterations in the post-translational modification and intracellular trafficking of clusterin in MCF-7 cells during apoptosis. *Cell Death Differ.* **10**, 914–927
- 48 Burden, D. A., Kingma, B. S., Froelich-Ammon, S. J., Bjornsti, M. A., Patchan, M. W., Thompson, R. B. and Oshero, N. (1996) Topoisomerase II–etoposide interactions direct the formation of drug-induced enzyme–DNA cleavage complexes. *J. Biol. Chem.* **271**, 29238–29244
- 49 Furuya, Y., Ohta, S. and Ito, H. (1997) Apoptosis of androgen-independent mammary and prostate cell lines induced by topoisomerase inhibitors: common pathway of gene regulation. *Anticancer Res.* **17**, 2089–2093
- 50 Gleave, M. E., Miyake, H., Zellweger, T., Chi, K., July, L., Nelson, C. and Rennie, P. (2001) Use of antisense oligonucleotides targeting the anti-apoptotic gene, clusterin/testosterone-repressed prostate message 2, to enhance androgen sensitivity and chemosensitivity in prostate cancer. *Urology* **58**, 39–49
- 51 Martin, S. S. and Leder, P. (2001) Human MCF10A mammary epithelial cells undergo apoptosis following actin depolymerization that is independent of attachment and rescued by Bcl-2. *Mol. Cell Biol.* **21**, 6529–6536
- 52 Akakura, K., Bruchovsky, N., Rennie, P. S., Coldman, A. J., Goldenberg, S. L., Tenniswood, M. and Fox, K. (1996) Effects of intermittent androgen suppression on the stem cell composition and the expression of the TRPM-2 (clusterin) gene in the Shionogi carcinoma. *J. Steroid Biochem. Mol. Biol.* **59**, 501–511
- 53 Reddy, K. B., Jin, G., Karode, M. C., Harmony, J. A. and Howe, P. H. (1996) Transforming growth factor β (TGF β)-induced nuclear localization of apolipoprotein J/clusterin in epithelial cells. *Biochemistry* **35**, 6157–6163
- 54 Scaltriti, M., Brausi, M., Amorosi, A., Caporali, A., D'Arca, D., Astancolle, S., Corti, A. and Bettuzzi, S. (2004) Clusterin (SGP-2, ApoJ) expression is downregulated in low- and high-grade human prostate cancer. *Int. J. Cancer* **108**, 23–30
- 55 Lapointe, J., Li, C., Higgins, J. P., van de Rijn, M., Bair, E., Montgomery, K., Ferrari, M., Egevad, L., Rayford, W., Bergerheim, U., Ekman, P. et al. (2004) Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 811–816
- 56 Han, B. H., De Mattos, R. B., Dugan, L. L., Kim-Han, J. S., Brendza, R. P., Fryer, J. D., Kierson, M., Cirrito, J., Quick, K., Harmony, J. A. et al. (2001) Clusterin contributes to caspase-3-independent brain injury following neonatal hypoxia–ischemia. *Nat. Med.* **7**, 338–343

Received 28 January 2004/15 April 2004; accepted 12 May 2004

Published as BJ Immediate Publication 12 May 2004, DOI 10.1042/BJ20040158