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Letter to the Editor

Ca^{2+} depletion induces nuclear clusterin, a novel effector of apoptosis in immortalized human prostate cells

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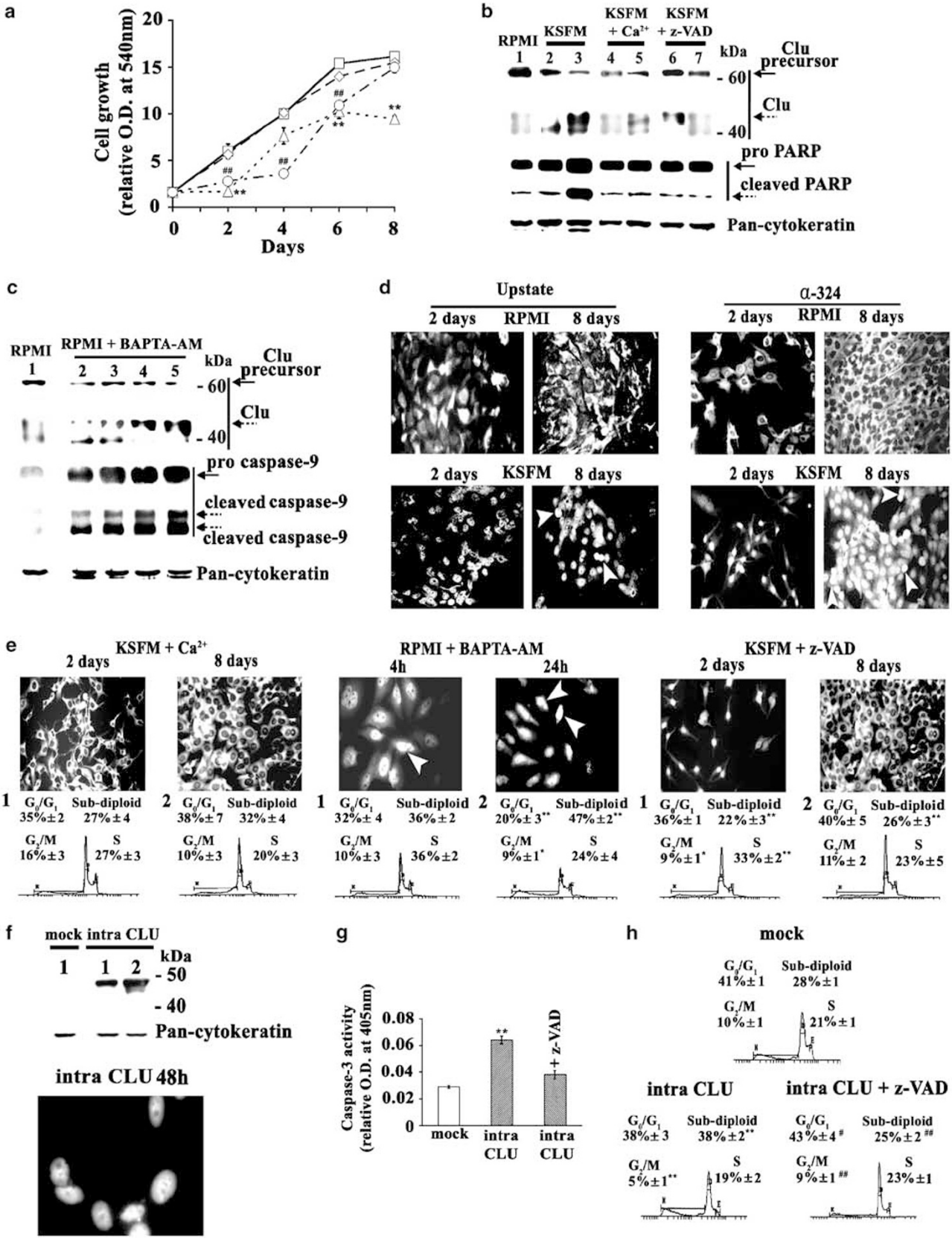
Dear Editor,

Clusterin (CLU) is a secreted heterodimeric glycoprotein that can be produced almost ubiquitously in mammalians tissues.[1] Its gene expression is subjected to complex regulation and can change enormously according to different stimuli.[2] Cloned and identified as the most potently induced gene in the regressing rat ventral prostate following androgen-ablation,[3] CLU was almost simultaneously characterized and isolated by different research groups working in widely divergent areas.[2] CLU is coded by a single copy gene, located on chromosome 8.[4] The gene codes for an initial precursor peptide glycosylated and cleaved into two α and β chains of 40 kDa each, held together by a unique five disulfide bond motif in the extracellular mature form.[1] This secreted form of CLU has been suggested to act as a molecular chaperone following stress-induced injury,[5] clearing extracellular debris.[6] However, it has been reported the existence of an inactive, cytoplasmic form of CLU produced by alternative splicing that is converted by ionizing irradiation to a truncated mature nuclear isoform,[7] which binds the Ku70/Ku80 complex in cell-free systems[8] inhibiting cell growth and survival[7] probably by a caspase-3-independent mechanism.[9] Other alternative CLU isoforms, produced either by exon skipping[10] or by post-translational modifications activated by apoptosis,[11,12] were recently described. These different isoforms of CLU have been suggested to be antiapoptotic[6,13] or proapoptotic.[7,10,12,14–16]

These controversial reports on the role of CLU might be related to specific proteomic profiles that are produced by different apoptotic stimuli (i.e. the general protein pattern of CLU and the relative ratio between different CLU isoforms). This might explain why CLU has been involved in a plethora of pathophysiological processes, including cell–cell and cell–matrix adhesion, cell differentiation, transformation, aging[17,18] and cancer,[19] but its biological role still remains to be clearly established. Reports suggesting that CLU may be a potential tumor suppressor gene include the finding that CLU suppresses NF-κB activity and the metastatic phenotype of neuroblastoma cells.[20] We have previously reported that CLU overexpression inhibits cell cycle progression of simian virus 40(SV40)-immortalized human prostate PNT2 and PNT1A epithelial cells.[21]

To further assess the role of CLU in apoptotic processes we have studied its expression pattern during the regulation of calcium homeostasis. Ca^{2+} is an important regulator of apoptosis and cell survival.[22,23,24] Both pathological increase of Ca^{2+} concentration in the cytosol compartment by inophores[25] and depletion of intracellular Ca^{2+} stores may trigger apoptosis by disrupting intracellular architecture and allowing effectors to gain access to their substrates.[24,26,27] Activation of apoptotic endonucleases[28] eliciting DNA cleavage and chromatin condensation has been well documented.[29,30,31] A tight buffering of intracellular Ca^{2+} is required for normal growth. In fact, apoptosis can be induced by Ca^{2+} mobilization from intracellular pools,[23,24,27] chelation of intracellular Ca^{2+} with 1,2-bis-(2-aminophenoxy)ethane-*N,N,N*^{1},*N*^{1}-tetra-acetic acid tetra-acetoxymethyl ester (BAPTA-AM)[28,29] or removal of extracellular Ca^{2+}.[32] Intracellular Ca^{2+} deficiency regulates gene expression[22,26] and induces apoptosis through caspases activation.[23,27,33] It has been shown that extracellular Ca^{2+} depletion reduces expression of CLU mRNA, but its proteomic profile has not been studied.[26] Thus, we decided to study the effect of Ca^{2+} depletion in prostate cells and its effect on CLU protein expression and localization.

We compared the cell proliferation rate of PNT1A cells grown under standard culture medium (RPMI plus 10% foetal bovine serum, FBS), keratinocyte serum-free complete medium[34] (KSFM, which has subphysiological concentrations of Ca^{2+}, 0.1 mM), or KSFM supplemented with 1.8 mM Ca^{2+} (Figure 1a). Under these condition, as previously reported,[35] KSFM caused a marked inhibition of cell growth rate when compared to standard culture conditions, inducing anoikis (cell detachment followed by chromatin condensation). Addition of either 1.8 mM Ca^{2+} or the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-flu-oromethylketone (z-VAD-fmk, 1 μM) to KSFM medium completely rescued PNT1A cells and prevented cell detachment and anoikis induction (Figure 1a). After 8 days of culture in KSFM medium supplemented with z-VAD-fmk, cells reached a density similar to that observed under standard conditions (Figure 1a). In PNT1A cells CLU expression was detected by Western blot assay with a commercially available mouse monoclonal antibody from Upstate Biotechnology (clone 41D). The prevailing form of CLU in the cells consists of a 65 kDa intracellular CLU precursor that is progressively converted to low-molecular weight and secreted CLU forms.[36] Upon KSFM incubation, the 65 kDa CLU precursor is rapidly converted to a 45 kDa CLU isoform that accumulates in parallel to poly(ADP-ribose)


a
Cell growth (relative O.D. at 540nm)
Days
b
RPMI
KSFM
KSFM + Ca2+
KSFM + z-VAD
kDa
Clu precursor
Clu
pro PARP
cleaved PARP
Pan-cytokeratin
c
RPMI
RPMI + BAPTA-AM
kDa
Clu precursor
Clu
pro caspase-9
cleaved caspase-9
cleaved caspase-9
Pan-cytokeratin
d
Upstate
α-324
2 days
RPMI
8 days
2 days
KSFM
8 days
e
KSFM + Ca2+
RPMI + BAPTA-AM
KSFM + z-VAD
2 days
8 days
4h
24h
G0/G1
Sub-diploid
G2/M
S
f
mock
intra CLU
kDa
Pan-cytokeratin
intra CLU 48h
g
Caspase-3 activity (relative O.D. at 405nm)
mock
intra CLU
intra CLU + z-VAD
h
mock
intra CLU
intra CLU + z-VAD

polymerase (PARP) activation (Figure 1b, KSFM 8-day, lane 3). Ca^{2+} administration blocked both the accumulation of the 45 kDa isoform and PARP activation (Figure 1b, KSFM + Ca^{2+}, 2 and 8 days, lanes 4 and 5, respectively). 2 days of z-VAD-fmk administration did not prevent the accumulation of the 45 kDa CLU isoform (Figure 1b, KSFM + z-VAD, lane 6). However, 8 days of treatment with z-VAD-fmk restored a CLU proteomic profile similar to that induced by Ca^{2+} administration (Figure 1b, KSFM + z-VAD, lane 7). To assess whether intracellular Ca^{2+} depletion would *per se* cause apoptosis, we cultured PNT1A cells in the presence of BAPTA-AM, a well-known intracellular Ca^{2+} blocker[26] that can trigger activation of caspases and apoptosis.[23,28,29,33] Cell proliferation was assessed by crystal violet staining method in the presence of 20 μM BAPTA-AM. As soon as 4 h after treatment, cells growth was significantly inhibited reaching about 55 and 78% inhibition at 24 and 48 h ($P<0.01$, data not shown), respectively, in strict association with chromatin condensation and fragmentation, cell rounding and detachment, hallmarks of anoikis (see Figure 1e, RPMI + BAPTA-AM). Consistently with the hypothesis that changes in the intracellular availability of Ca^{2+} could trigger anoikis in PNT1A cells, a switch to the 45 kDa isoform of CLU was already observed 4 h after the beginning of BAPTA-AM administration, a time when caspase-9 activation started to be evident (Figure 1c). At 24–48 h-time of BAPTA-AM administration, when the decrease in cell proliferation was massive (data not shown), PNT1A cells showed the highest level of 45 kDa CLU isoform accumulation (Figure 1c, RPMI + BAPTA-AM, lanes 4 and 5).

Immunocytochemical analysis of PNT1A cells showed subcellular localization of CLU under different experimental conditions. We compared the commercially available mouse monoclonal anti-CLU antibody and an affinity-purified polyclonal anti-human CLU antibody (α-324) that we have raised against an epitope located on the α-subunit of human CLU protein at position 324–341 (Accession Number X14723, cfr. legend to Figure 1). Experiments were conducted in parallel with cells grown in standard RPMI or in KSFM medium (Figure 1d). Western blot analysis using the α-324 antibody confirmed the detection of the 45 kDa nuclear CLU isoform in cells grown in KSFM medium (data not shown). Under standard conditions, CLU was detectable in the cytoplasm (Figure 1d, RPMI 2 and 8 days). Conversely, CLU was detected in the nucleus already 2 days after KSFM-growth (Figure 1d, KSFM). Detection of CLU in the nucleus preceded cell morphological changes. After 8 days in KSFM medium, nuclear CLU accumulated into apoptotic bodies (Figure 1d, KSFM). The comparison between the two antibodies showed that α-324 gave better results when compared to the commercially available mouse monoclonal anti-CLU antibody (Figure 1d, compare α-324 and Upstate). When KSFM was supplemented with 1.8 mM Ca^{2+}, CLU was clearly localized in the cytoplasm (Figure 1e, KSFM + Ca^{2+}, 2 and 8 days), and cells growth rates (Figure 1a) and cell cycle progression (Figure 1e, subpanels 1 and 2) were identical to that observed under standard growth conditions (2 days RPMI). As expected, BAPTA-AM administration caused a dramatic reduction in cell growth (data not shown) and viability (Figure 1e, RPMI + BAPTA-AM, subpanels 1 and 2). Fluorescence activated cell sorting (FACS) analysis revealed a progressive loss of the G_0/G_1 fraction in concomitance to an increase of the subdiploid peak (Figure 1e, RPMI + BAPTA-AM, subpanels 1 and 2). When cells were incubated in KSFM + z-VAD-fmk for 2 days, CLU was detected into the nuclei (Figure 1e, KSFM + z-VAD, 2 days), but DNA fragmentation was blocked (Figure 1e, KSFM + z-VAD, subpanels 1 and 2). At 8-day culturing, clusterin was almost completely detected into the cytoplasm (Figure 1e, KSFM + z-VAD, 8 days), a time when cells were completely rescued and cell growth rate was very similar to controls and significantly higher than that observed in KSFM alone (Figure 1a). Anoikis induction and

Figure 1 Effects of extracellular and intracellular Ca^{2+} depletion, Ca^{2+} supplementation or z-VAD-fmk administration on cell growth and survival of PNT1A cells. The SV40-immortalized human prostate epithelial cell line PNT1A was previously established and characterized.[40] (**a**) Cell growth of PNT1A cells with standard RPMI culture medium supplemented with 10% FBS and 1% L-glutamine (□), serum-free, low-Ca^{2+} KSFM culture medium supplemented with epidermal growth factor (EGF, 5 ng/ml), bovine pituitary extract (BPE, 50 μg/ml) and 1% L-glutamine (△) or KSFM culture medium supplemented with either 1.8 mM Ca^{2+} (◇) or 1 μM z-VAD-fmk (○). Cell proliferation was assessed using the crystal violet dye method. Optical densities (OD) are expressed as the mean ± standard deviations (S.D.) of three independent experiments ($n=3$), and error bar indicate standard deviations. Statistical significance was calculated by the Student's *t*-test (**$P<0.01$) (##$P<0.01$). (**b**) Accumulation of a 45 kDa CLU isoform during anoikis induction. CLU proteomic profile was studied by Western blot analysis in cells grown with standard RPMI medium for 2 days (lane 1), or in KSFM medium for 2 or 8 days (lanes 2 and 3), or KSFM + Ca^{2+} (lanes 4 and 5) or KSFM + z-VAD (lanes 6 and 7). Equal amounts (50 μg) of total cell protein extracts were loaded and resolved by SDS-PAGE and then subjected to Western blot analysis with the indicated antibodies. (**c**) Western blot analysis showing CLU expression in standard RPMI medium after 24 h (lane 1) or in the presence of BAPTA-AM after 4 h (lane 2), 16 h (lane 3), 24 h (lane 4) or 48 h (lane 5). Blotted membranes were also immunostained with polyclonal anti-human caspase-9 antibody (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Expression of Pan-cytokeratin shows equal loading of the lanes. (**d**) Immunocytochemistry showing CLU localization in PNT1A cells grown in RPMI or KSFM medium was carried out with monoclonal 41D antibody (Upstate; 1 : 50) or the α-324 antibody (1 : 80). The α-324 polyclonal antibody was obtained by injecting the peptide RRELDESLQVAERLTRKY (aa 324–341) into rabbits. Cells were fixed and then incubated with the upstate or α-324 antibodies. Secondary antibodies were anti-mouse Alexa Fluor™ (568-red fluorescence, 1 : 200) or anti-rabbit-Alexa Fluor™ (568-red fluorescence, 1 : 200). Digital images were acquired with a color digital camera. Magnification: × 20. (**e**) Ca^{2+} or z-VAD-fmk administration prevents whereas calcium chelation induces accumulation of nuclear CLU and anoikis in PNT1A cells. Cells were cultured in KSFM + Ca^{2+} for 2 and 8 days, fixed, and subjected to immunocytochemistry (magnification: × 20), as reported above, with the α-324 antibody. Cells were cultured with 20 μM BAPTA-AM for 4 or 24 h, fixed and subjected to immunocytochemistry (magnification: × 40). Cells were grown in the presence of KSFM + z-VAD for 2 or 8 days, fixed, and subjected to immunocytochemistry (magnification: × 20). FACS analysis of cell cycle progression was performed as previously described.[21] Values are the mean of cell population (% of total) ± S.D. ($n=5$). Significant differences *versus* 2 days RPMI (G_0/G_1, 35% ± 1; S, 20% ± 3; G_2/M, 12% ± 3; Subdiploid, 33% ± 5) are indicated (*$P<0.05$, **$P<0.01$). Effects of overexpression of intra-CLU on cell proliferation and apoptosis. (**f**, top panel) Cells were transfected with either pIRES-hyg1 containing the intra-CLU cDNA, or empty pIRES-hyg1 expression vector (mock control).[41] Cells were harvested after 24 h (lanes 1) and 48 h (lanes 2), and subjected to Western blot analysis with the α-324 antibody. (**f**, bottom panel) Nuclear localization of intra-CLU is shown after transfection and immunohistochemistry. Magnification: × 63. (**g**) Caspase-3 activity in PNT1A cells 48 h after transfection. The CPP32/Caspase-3 Colorimetric Protease Assay kit (MBL, International Corporation, Watertown, MA, USA) was used according to the manufacturer's instructions. Values are mean ± S.D. ($n=3$); **$P<0.01$. (**h**) Cell cycle analyses of transfected PNT1A. Values are mean ± S.D. ($n=5$) (#$P<0.05$; ** or ##$P<0.01$)

PARP activation were blocked at 8-day KSFM incubation with z-VAD-fmk (Figure 1b and e), suggesting that nuclear translocation of 45 kDa clusterin primes cells to apoptosis.

To directly demonstrate that nuclear localization of CLU can induce anoikis, we transfected PNT1A cells with an expression vector containing a shortened CLU cDNA (intra-CLU) that starts from nucleotide 152 and therefore lacks the hydrophobic secretion signal sequence. Following transient transfection, PNT1A cells showed a detectable suppression of cell proliferation (data not shown) and decreased viability in association to a progressive accumulation of intra-CLU (49 kDa, Figure 1f, top). Intra-CLU accumulated in the nuclei of about 75% of transfected cells (Figure 1f, bottom). Notably, expression of nuclear CLU induced a significant increase in caspase-3 activity (Figure 1g), DNA fragmentation (Figure 1h) and cell detachment. Nuclear CLU caused cell death by activation of caspase-dependent apoptosis. In fact, z-VAD-fmk significantly rescued cells from anoikis and DNA fragmentation (Figure 1g and h, intra-CLU + z-VAD *versus* mock).

We report here that calcium (Ca^{2+}) deprivation causes translocation of a 45 kDa CLU isoform to the nucleus in human prostate epithelial cells, leading to inhibition of cell proliferation and caspase-cascade-dependent anoikis. Our data show that accumulation of nuclear CLU caused induction of apoptosis and anoikis in prostate cells, and that conditions restoring normal cell growth and survival also cause re-localization of CLU in the cytoplasm. In fact, early before induction of anoikis, we observed translocation of a 45 kDa CLU isoform in the cell nuclei that was prevented by Ca^{2+} or z-VAD-fmk supplementation, leading to cell rescue. Importantly, transient overexpression of an intracellular, not secreted, form of CLU accumulating into the nucleus caused cell growth inhibition and anoikis in the absence of Ca^{2+} deprivation. This effect was also caspase-dependent. In any condition studied, cell demise was strictly associated with translocation of CLU to the nucleus. Taken together, these data suggest that anoikis-death by Ca^{2+} deprivation is accompanied by nuclear translocation of a 45 kDa CLU and that a nuclear only form of CLU appears to recapitulate death by Ca^{2+} deprivation, suggesting a putative causative link between Ca^{2+} depletion, CLU nuclear translocation and cell death. CLU intracellular trafficking can now be linked to Ca^{2+} signalling pathway, resulting in regulation of survival and proliferation of androgen-dependent prostate epithelial cells. Moreover, the data here presented and previous results showing that CLU is downregulated during prostate cancer progression[19,37] support the hypothesis that CLU is a tumor-suppressor gene for the prostate. The finding that calreticulin, an intracellular Ca^{2+}-binding protein, is regulated by androgens,[38] and that inhibition of spontaneous and androgen-induced prostate growth by a vitamin D analog involves CLU,[39] also supports the hypothesis that a causative link between these pathways may exist.

Identification of the molecular mechanisms involved in the production and processing of intra-CLU will help in the future the molecular characterization of prostate cancer, one of the most diffuse and elusive type of cancer.

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