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Role of clusterin in cell adhesion during early phases of programmed cell death in P19 embryonic carcinoma cells

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Abstract

This study explored the role of clusterin in mechanisms of cell adhesion and apoptosis in P19 embryonic carcinoma cells. We found that serum deprivation induced transient but dramatic elevation in cell adhesion strength to the culture substrate and eventually led to apoptotic cell death. The time course of cell-adhesion increase overlapped temporally with the elevation of clusterin mRNA (peak 8 h after serum deprivation). The coincidental elevation of clusterin expression and cell adhesion strength preceded the schedule of apoptotic cell death. Clusterin antiserum partially antagonized cell adhesion, but did not modify the course of apoptosis. These data suggest that clusterin expression may partially control cell adhesion with no influence on apoptosis in P19 cells, under defined conditions.

Keywords: Apolipoprotein J; Sulfated glycoprotein-2; Testosterone repressed message-2; Clusterin expression; Apoptosis; Serum deprivation-induced cell death

1. Introduction

Clusterin is an amphiphilic glycoprotein found in many tissues and fluids, for which several possible functions have been proposed. Originally named for its activity in aggregating (clustering) spermatozoa [1], clusterin has numerous other acronyms, derived from various attributes and associations, that include complement lysis inhibition (CLI, SP-40,40) [2,3] and apolipoprotein transport (apoJ) [4]. It is also known as sulfated glycoprotein 2 (SGP-2) [5].

When identified as a testosterone repressed message-2 (TRPM-2), its induction was associated with apoptosis in certain epithelial and mesenchymal cells, e.g., in the prostate after castration [6], kidney ducts after ureteral obstruction [7] and with neurodegeneration [8]. Although clusterin is often increased in regressing or injured tissues, it cannot be regarded as a marker of cell death because of its constitutive expression by many types of healthy cells in several tissues and organs, including the brain [9,10]. Moreover, in several in vivo models of cell death, clusterin mRNA was shown to be selectively upregulated in surviving cells [11–13] while clusterin depletion was shown to enhance injury [14]. There is also evidence that clusterin may be involved in mechanisms of cell-adhesion [15,16] which may be pertinent to its role in tissue reorganization after injury [17,18].

The precise biological function(s) of clusterin in mechanisms of cell death/survival is unknown.

In this study we further examined the role of clusterin in P19 embryonic carcinoma cells during responses to serum deprivation. In these conditions we have previously shown that P19 cells undergo apoptotic cell death, showing DNA fragmentation, partially inhibited by cycloheximide, and the typical changes in nuclear morphology [19]. Here we describe a transient increase in cell adhesion in the same cells and a coincidental regulation of clusterin expression, which preceded the onset of apoptotic nuclear fragmentation. Clusterin antiserum partially antagonized the changes in cell adhesion, but failed to prevent the morphological changes characteristic of apoptosis. This study suggests alternative role(s) of clusterin during response to stimuli leading to apoptosis, under defined conditions.

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2. Materials and methods

2.1. Cell culture

P19S1801A1 (P19) cell culture and media composition were as previously described [19]. Briefly, cells were dissociated with pancreatin (0.4 × U.S.P.; Gibco, Paisley, UK), washed once with complete medium (Dulbecco's Modified Eagles Medium plus 10% fetal calf serum (FCS)), twice with serum-free medium and plated in 25 cm^2 tissue culture flasks (Falcon Becton Dickinson, Lincoln Park, New Jersey) at a final density of 10^4 cells/cm^2 in 5 ml of serum-free or complete medium. Cells were examined for cell adhesion strength to culture substrate (plastic), apoptosis (nuclear morphology) and clusterin expression (mRNA and protein).

When indicated, flasks were coated overnight with human fibrinogen (grade L, Ortho Diagnostic Systems, Milan, Italy) 250 μg/ml in PBS with Ca^{2+} and Mg^{2+}, then 1 h with 1% bovine serum albumin, followed by three washes with PBS.

2.2. Cell adhesion assay

Quantitation of cell adhesion was made as previously described, with minor modifications [20]. Briefly, after removal of culture medium, cells were incubated with 0.5 ml pancreatin for a defined time (usually 3 min) at 37°C in a water bath, then 1 ml of complete medium was gently distributed over the surface of the culture to inactivate pancreatin and collect detached cells (with no shaking). The number of detached cells was determined by counting cells that excluded Trypan blue in an aliquot of the 1.5 ml suspension, in a standard hemocytometer. This procedure is sufficient to detach about 50% of the cells in normal control P19 cultures in 1 min, 70% in 3 min and 100% in 5 min. A lower number of detached cells was indicative of a higher number of cells that were resistant to proteolitic detachment and, by consequence, of a higher strength of adhesion to substrate.

2.3. Nuclear morphology

P19 cells were collected by gently pipetting, resuspended in serum-free medium and seeded on microscope slides by cytospin centrifugation (Heraeus, Handu, Germany) at 600 rpm for 5 min. They were then fixed in Carnoy solution (methanol:acetic acid, 3:1) for 1 h, stained with Hoechst 33258 (0.1 μg/ml in phosphate-buffered saline) for 1 h at 37°C, washed with tap water for 1 h, air-dried and mounted. Slides were examined with a fluorescence microscope (Zeiss, Oberkochen, Germany) at an excitation wavelength of 365 nm, counting at least 500 cells in triplicate preparations [19].

2.4. RNA analysis (Northern blot)

Total RNA was extracted from individual samples with a standard guanidinium thiocyanate-CsCl method [21]. Total RNA (10 μg) was electrophoresed on denaturing (0.2 M formaldehyde) agarose gels in MOPS buffer (MOPS 40 mM, Na-acetate 10 mM, EDTA 1 mM, pH 8) for 5 h at 70 Volts, and blotted to nylon membrane (Gene Screen™ Plus, DuPONT, Dreieich, Germany) in 10× SSC (NaCl 1.5 M, sodium citrate trisodium salt dihydrate 15 mM, pH 7). Antisense [^{32}P]cRNA for clusterin was transcribed from linearized cDNA transcription vector; the rat clusterin cDNA clone was a gift from M. Griswold (University of Washington, Pullman). RNA blots were hybridized with 10^6 CPM/ml. Blots were washed to a final stringency of 0.2 × SSC, 0.2% SDS at 72°C, then exposed to Kodak XAR-5 X-ray film (Eastman Kodak, Rochester, NY) with intensifying screen at −70°C. Northern blot hybridization of P19 total RNA with labelled clusterin cRNA detected the expected single mRNA band of 2.0 kb [10,22,23]. Integrated optical densities were collected by computerized video densitometry (Image Measure, Microscience, Federal Way, WA). Data were analyzed by ANOVA followed by LSD post hoc analysis.

2.5. Protein analysis (Western blot)

P19 cells, cultured in serum-free medium were collected, centrifuged at 150 × *g* and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.5 mM benzamide, 0.01% soybean trypsin inhibitor). Cells were lysed by 5 freeze/thaw cycles, centrifuged at 13 000 × *g* for 15 min at room temperature, then pellets and supernatants were separated. The pellet is a fraction rich in plasma membranes, nuclei, mitochondria, Golgi vesicles and lysosomes, and the supernatant includes cytosol and microsomes [24]. Protein content was determined by Lowry's method [25]. Proteins (20 μg/sample) and conditioned media (concentrated 40-fold on 30 kDa cut-off Centricon concentrators (Amicon, Beverly, MD) were resuspended in 62.5 mM Tris-HCl, pH 6.8, containing 100 mM DTT, 8 M urea, 5% (w/v) glycerol and 0.01% bromophenol blue tracking dye and resolved in 10% SDS-polyacrylamide gels by the discontinuous method of Laemmli [26], as modified by Douglas and Butow [27]. Gels were blotted onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) for 3 h at 70 V. Membranes were stained with Ponceau-S, blocked in Tris-buffered saline (TBS) containing 0.075% Tween and 0.1% bovine serum albumin for 1 h at room temperature, and immunoreacted with a rabbit anti-rat clusterin antiserum (1:500 dilution from stock, from M. Griswold) [10,22,28]. Immunoreactivity was visualized by chemiluminescence (ECL, Amersham) and molecular weights (kDa) were calculated from standards (Bio-Rad, Richmond, CA). The

same clusterin antiserum (IgG fraction) was used for immunoblotting and adhesion assays.

3. Results

Changes of cell adhesion, apoptosis and clusterin expression were examined in P19 cells at each time indicated after plating in serum free or complete medium. Fig. 1A,B shows the morphology of P19 cells 24 h after plating in conditions of complete (A), or serum free (B) medium. At this time all cells, also in B, were attached to culture substrate (plastic) and were viable, as assessed by Trypan blue exclusion (not shown). In Fig. 1C we show representative nuclei stained with Hoechst 33258, with the typical apoptotic morphology. The proportion of apoptotic nuclei in serum-deprived cells was less than 2% by 8 h after plating, and reached 15% and 25% by 24 and 36 h, respectively (not shown) [19].

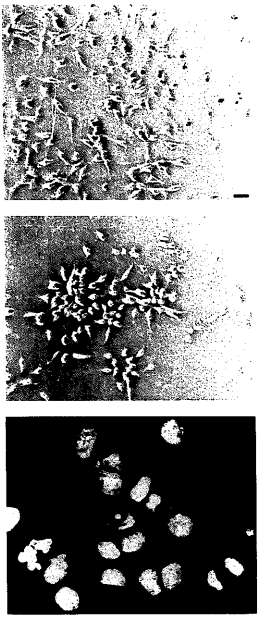

Fig. 1. Morphology of P19 cells. Phase-contrast photomicrographs of P19 cells either in complete (A) or in serum-free (B) medium 24 h after plating. At this stage cells in serum-free medium are still attached to substrate, but are organized in clusters. (C) Nuclear morphology of cells, stained with Hoechst 33258, 36 h after plating in serum-free medium. Nuclei presenting chromatin condensation and fragmentation characteristic of apoptosis are visible, together with normal nuclei, with dispersed chromatin and larger in size. Original magnification 100× in A and B, 400× in C. Bar 50 μm in A and B, 8 μm in C.

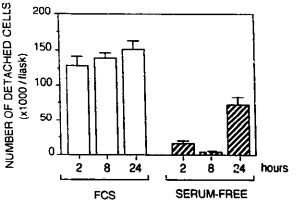

Fig. 2. Time course of change of P19 cell adhesion to substrate in response to serum deprivation. Cells were plated at the density of 2.5×10^5/flask in either complete (fetal calf serum, FCS) or serum-free medium. Cell adhesion strength was quantified by counting the number of cells that could be detached by a limited proteolytic treatment (3-min pancreatin incubation, see Section 2.2) at 2, 8 and 24 h after plating in complete (FCS) or in serum-free medium. A lower number of detached cells was indicative of a higher number of cells that were resistant to proteolitic detachment and, by consequence, of a higher strength of adhesion to substrate. Means ± S.D., $n = 4$ per group.

P19 cells in serum-free medium (Fig. 1B) showed a non-homogeneous distribution as compared to cells in complete medium (Fig. 1A), and were organized in clusters. We therefore hypothesized that, despite the same attachment, they could show differences in adhesion. We found that at 2 and 8 h after plating in serum-free conditions, P19 cells adhered more strongly to the culture substrate when compared with cultures in complete-medium (Fig. 2). Cell adhesion was quantified by counting the number of cells that could be detached by a limited proteolytic treatment (see Section 2.2 and Ref. [20]). P19 cells reached maximum cell adhesion by 8 h after plating in serum-free medium, returning toward control levels by 24 h (Fig. 2). The time course of cell adhesion increase was therefore considerably faster as compared with the appearance of apoptotic figures in the same cells (see above and Ref. [19]).

The same difference in adhesion, although to a lower extent, was observed when cells were cultured on fibrinogen-coated flasks: the number of cells detached by 3 min incubation with pancreatin, by 8 h after plating, was 282 400 ± 46 300 and 165 300 ± 27 300 for control and serum free cells, respectively (mean ± S.D., $n = 4$, $P < 0.01$, Student's t-test).

The time course of changes of cell adhesion coincided with the peak elevation of clusterin mRNA (Fig. 3A,B). Northern blot hybridization of total RNA from P19 cells with rat cRNA probes detected a single 2.0 kb mRNA

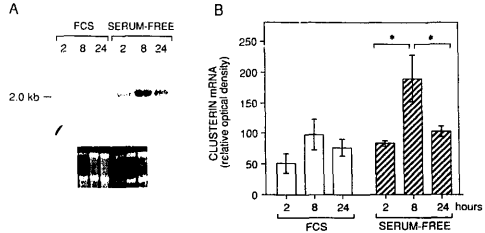

Fig. 3. Time course of clusterin mRNA changes in P19 cells following serum deprivation. In A, the upper panel shows clusterin mRNA Northern blot hybridization signal of total RNA from P19 cells 2, 8 and 24 h after plating in complete (FCS) or in serum-free medium. In the lower panel, ethidium bromide staining controlled for RNA loading. In B, time course of clusterin mRNA change as assessed by Northern blot assay. Mean ± S.E.M. of the results from 4–5 independent determinations. * $P < 0.05$.

species, as previously found in rat and human brain [10] (Fig. 3A). As assessed by Northern blot hybridization assay, the schedule of clusterin mRNA change, overlapped temporally with the schedule of cell adhesion showing a coincidental peak elevation by 8 h after plating in serum-free conditions (Fig. 3B).

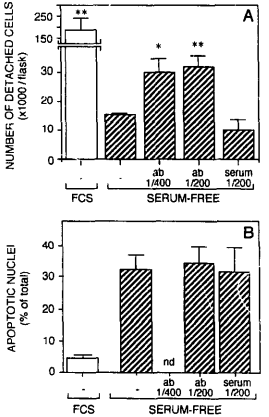

Fig. 4. Inhibition of cell-adhesion strength, but not of apoptosis by clusterin antiserum. In A, cells were plated either in complete medium (FCS) or serum-free medium supplemented with saline (–), two dilutions of a rabbit anti-rat clusterin polyclonal antiserum (ab 1/400 and 1/200), or preimmune rabbit serum (serum 1/200). Adhesion strength was then measured as the number of cells that could be detached by proteolytic treatment, 8 h after plating. In B, the number of apoptotic nuclei was determined 36 h after plating. Mean ± S.D., $n = 4–5$ per group. ** $P < 0.01$ and * $P < 0.05$ vs the group with no antiserum (–).

Using rabbit anti-rat (IgG) clusterin antiserum, immunoblots of P19 cells under reducing conditions revealed two bands of about 36 and 39 kDa (not shown), in agreement with the electrophoretic pattern of the processed dimeric forms of rat brain (and serum) clusterin [10,22]. Clusterin immunoreactivity was detectable in the cytosolic fraction by 2 and 8 h and transiently, in the membrane fraction by 8 h after plating in serum-free conditions. No clusterin immunoreactivity was found in both fractions by 24 h after plating and in the concentrated (40-fold) conditioned media at any time examined (2, 8 and 24 h) (not shown).

To assess the role of clusterin in P19 cells during conditions of serum deprivation, we examined the effect of the same clusterin antiserum on cell adhesion strength and apoptosis. While clusterin antiserum (1:400, 1:200 dilutions) partially reduced cell adhesion strength by 8 h (Fig. 4A), it failed to block apoptosis by 36 h after plating (Fig. 4B). Control pre-immune rabbit serum was negative on both conditions (Fig. 4A,B). The increase in the number of detachable cells obtained by clusterin antiserum in three independent experiments was 192 ± 14% (mean ± S.D.) of untreated, serum-deprived cells.

4. Discussion

This study explored the role of clusterin in mechanisms of cell adhesion and apoptosis and extends previous studies on mechanisms of apoptotic cell death in P19 carcinoma cells during responses to serum deprivation [19]. While there is evidence that clusterin expression is elevated in several models of neurodegeneration [22,28], tissue regression [11] and apoptosis [6], the precise biological function(s) of this protein is far from being understood.

In our study we show that clusterin antiserum partially antagonized the increase in cell adhesion that occurred in serum deprived P19 cells prior to the occurrence of nuclear

fragmentation. However, it failed to affect apoptosis which may also be due to the fact that the clusterin antiserum did not have access to intracellular mechanisms leading to apoptosis. This study suggests therefore that clusterin could control cell-adhesive mechanisms, irrespective to conditions leading to apoptosis in the same cells. This perspective is consistent with the discrepancy on the role of clusterin in cell death (apoptosis) and/or tissue regression/remodelling, reported in several experimental models, in vivo or in vitro (see Section 1). Moreover, recent data show that clusterin depletion rather than its expression is associated with cell death, and suggest that clusterin plays a role in the protection against death [11–14,29].

Since clusterin is a secretory protein (in testis, adrenal glands, as well as astrocytes, etc., summarized in Ref. [10]), the absence of clusterin in the conditioned media is puzzling. However, it does not imply that clusterin is not secreted by P19 cells. We cannot exclude that the protein is present in the cell matrix. Additionally, it has recently been reported that glycoprotein 330, a protein related to the low density lipoprotein receptor, can bind clusterin and mediate its internalization in F9 teratocarcinoma cells [30] Clusterin may therefore be secreted and then rapidly bound in serum-deprived P19 cells too. Since our membrane-enriched fraction also contains organelles such as lysosomes, our data are compatible both with the presence of clusterin at the cell surface and with its internalization.

Clusterin (GP80) has also been attributed a cell-adhesive role during tissue remodelling as shown in the slime mold *Dictyostelium discoideum* during development [31,32]. Moreover, during response to experimental brain lesions, the elevation of clusterin expression is also localized in regions where synaptic remodelling occurs [33,34]. Thus, clusterin could also play a role in synaptic reorganization through its cell adhesion properties.

The time course of clusterin mRNA increase paralleled the increase of adhesion strength, both peaking 8 h after serum deprivation. At this time after plating in serum free conditions, the increase in adhesion strength was significantly lowered by the addition of clusterin antiserum at two concentrations, which were equally effective, suggesting a ceiling effect. Although the inhibition of cell adhesion by clusterin antiserum was only partial, it was consistently found in three independent experiments, suggesting a significant role of clusterin in cell adhesion. We note that other factors besides clusterin might control cell adhesion in P19 cells.

In conclusion our study indicates that clusterin does not play a pivotal role in the causative pathways that lead to cell death, but it may contribute to the adhesive interactions with the substrate. Moreover our study in P19 cells raises new questions about alternative functions of clusterin in regressing tissues. P19 cells under condition of serum deprivation provide an invaluable in vitro model to further assess the role of clusterin in mechanisms of cell adhesion and apoptotic cell death in the same cells.

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