Clusterin (SGP-2) gene expression is cell cycle dependent in normal human dermal fibroblasts

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Abstract In confluent human dermal fibroblasts brought to quiescence (G_0) by serum starvation, the S phase peaked at 24 h after serum re-addiction and G_2/M phase peaked at 36 h. This was confirmed by titration of h-gas1 mRNA (a marker of G_0 phase) and histone H3 (a marker of S phase). Clusterin mRNA accumulation progressively increased in cells proceeding to confluence after seeding and to quiescence upon serum starvation, and peaked at around G_0, in parallel with h-gas1 mRNA. At 6 h (roughly G_1 phase) clusterin transcript formed a second peak, followed by a gradual decrease until 36 h. Correspondence of clusterin protein accumulation to its mRNA occurred solely with regard to the G_0 peak but not to the second one. The possible meaning of the cell cycle related clusterin gene expression is discussed.

Key words: SGP-2; Clusterin; Human dermal fibroblast; Cell cycle; Human growth arrest specific protein 1; Histone H3

1. Introduction

Clusterin is known as a heterodimeric secreted glycoprotein of about 80 kDa present in most animal tissues and body fluids. It was originally described as the major rat Sertoli cell secretory product [1] and as the major glycoprotein in ram rete testis, endowed of cell-aggregating properties (hence the name of clusterin) [2]. Subsequently, clusterin gene was found to be the major up-regulated gene in the rat ventral prostate undergoing tissue regression after androgen ablation [3]. Under normal conditions, it is present at very low levels in this gland because it is solely expressed in the cuboidal (atrophic) cells, a minor population of prostate epithelial cells [4,5]. In the same organ, the abundance of clusterin mRNA undergoes a marked, almost linear, increase with ageing [5,6].

The human counterpart of the clusterin gene is present as a single copy in the human genome and localizes to chromosome 8 (8p21) [7].

Several synonyms/acronyms have been used to name this gene/protein, because of the different biological models in which it appears to play a role. Thus, the name 'clusterin' has been proposed in accordance with an international consensus regarding terminology [8]. Ever since, this highly conserved protein has been found to be involved in many biological processes, including apoptosis, cell adhesion and cell-cell interactions, organogenesis, cell differentiation and transformation, membrane lipid remodelling and transport, reproduction, complement regulation, oxidative stress, senescence and many others (reviewed in [8,9]). Differential regulation of this gene was shown to occur also in many disease states including renal diseases, atherosclerosis, neurodegenerative diseases, infertility, myocardial infarction and cancer [9].

Early investigations suggested a direct association of clusterin gene expression with programmed cell death [10]; however, recent reports indicate that clusterin is up-regulated in different cell types surviving death [11–13]. The latter observation may correlate with the enhanced levels of clusterin mRNA found in the prostate of the aging rat [5,6] and rat embryo fibroblasts undergoing senescence upon T antigen inactivation [14], suggesting a role for this gene in preventing cell death.

Very recently, however, it has been reported [15] that, upon androgen ablation, the different clusterin species found in the rat prostate specifically accumulate in the epithelial cells undergoing apoptosis.

We have shown previously that, in several biological systems under the action of agents causing either tissue proliferation or regression, clusterin gene expression undergoes changes that are synchronous but opposite to those of ODC (EC 4.1.1.17) [4,16]. Since ODC is a well known cell cycle dependent gene that peaks in late G_1/early S phases [17,18], we addressed the question of whether in primary cultures of synchronized human dermal fibroblasts the expression of clusterin gene undergoes cell cycle dependent changes during the first round of synchronized cell replication.

2. Materials and methods

2.1. Cell cultures and flow cytometry analysis

1.2×10^6 dermal fibroblasts isolated from skin biopsies of healthy subjects were plated in DMEM supplemented with 10% FCS and antibiotics in 75 cm^2 flasks [19]. To study the proliferative response, confluent cultures were 'serum starved' for 14 days in DMEM containing 1% FCS and stimulated by addition of fresh medium supplemented with 20% FCS. The proliferation stages of the cells at different times during cell cycle progression were assessed by two-parameter flow cytometry analysis. This was performed after dual staining: with PI (red fluorescence) for total DNA content, and with anti-BrdU/FITC (green fluorescence) after BrdU incorporation into the newly synthesized cellular DNA, for the specific labelling of cells in S phase. In the latter instance, 10.0 μM BrdU (from Sigma-Aldrich, Italia S.r.l.) was added to the cultures at the indicated times, followed by incubation at 37°C for 30 min; cells were then harvested by trypsinization and washed twice in 1×PBS, and the DNA was denaturated by incubation with 2.0 N HCl/Triton X-100 for 30 min at room temperature to produce single-strand molecules. After neutralization

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Abbreviations: SGP-2, sulfated glycoprotein 2 (clusterin); FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; BrdU, 5′-bromo-2′-deoxyuridine; PI, propidium iodide; h-gas1, human growth arrest specific protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PHA, phytohemoagglutinin; ODC, ornithine decarboxylase

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with 0.1 M $Na_2B_4O_7$ pH 8.5, followed by resuspension in 0.5% Tween-20 plus 1.0% BSA in 1×PBS, 10 μl of anti-BrdU/FITC (Caltag Laboratories, Burlingame, CA, USA) per 1×10^6 cells was added, and cells were incubated for 30 min at room temperature. After a further washing in Tween/BSA/PBS, cells were stained for DNA content with 5.0 μg/ml of PI and immediately analyzed by flow cytometry.

2.2. Northern hybridization

Total cellular RNA was extracted from cell cultures grown under the above conditions, at the time indicated, using RNAfast (Molecular Systems, San Diego, CA, USA). Ten μg aliquots were electrophoresed in a 1% agarose-formaldehyde gel, blotted on Hybond-N nylon membranes (Amersham, Italia S.r.l) and then hybridized to the specific SGP-2 (clusterin) or GAPDH cDNA probes, labelled by random priming ^{32}[P]-dCTP incorporation as previously indicated [6]. The cDNA fragments were excised out by digestion with the appropriate restriction enzymes and purified from agarose gel by using the Jetquick Gel Extraction Kit (Genomed GmbH, Germany). The h-gas1 cDNA, kindly donated by Dr. G. Del Sal [20], and the histone H3 full-length cDNA, kindly donated by Dr. G. Stein [21], were purified, labelled and used as specific probes following the same procedures. Quantitation of the autoradiograms was then obtained by densitometric scanning (LKB Ultrascan XL densitometer).

2.3. Western blots

At the times indicated, cells obtained from cell cultures treated as above were harvested, washed twice with cold PBS and directly lysed at 100°C for 10 min in SDS-PAGE loading buffer. The total protein extract, equivalent to 4×10^5 cells, was then loaded on each lane and resolved by electrophoresis on 10% polyacrylamide gel. Clusterin immunoreactive bands were detected with the BM Chemiluminescence Blotting Substrate (POD) (Boehringer Mannheim GmbH, Germany) using polyclonal anti-rat clusterin antibodies (UPSTATE Biotechnology, Lake Placid, NY, USA).

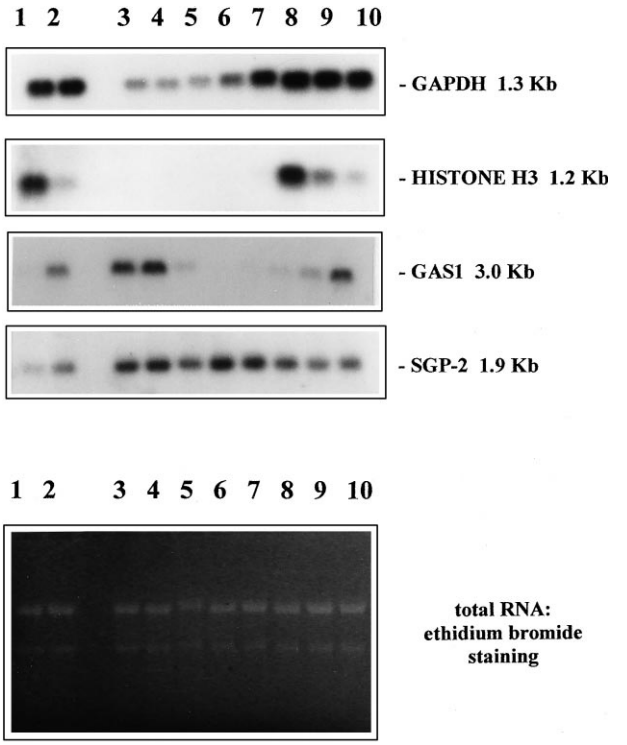

Fig. 1. Representative Northern blot experiments on total RNA extracted from cells harvested at the following times: lane 1, 2nd day after plating; lane 2, 3rd day after plating (cells at confluence); lane 3, 14 days after serum starvation (quiescence state). From lane 4 to lane 10: 1 h, 3 h, 6 h, 12 h, 24 h, 36 h and 48 h after serum stimulation, respectively. The specific radioactive probes used for hybridization and the size of the specific mRNAs detected are indicated. The ethidium bromide staining of the same RNA specimens, separated on agarose gel, is shown for comparison (10 μg of total RNA was loaded on each lane).

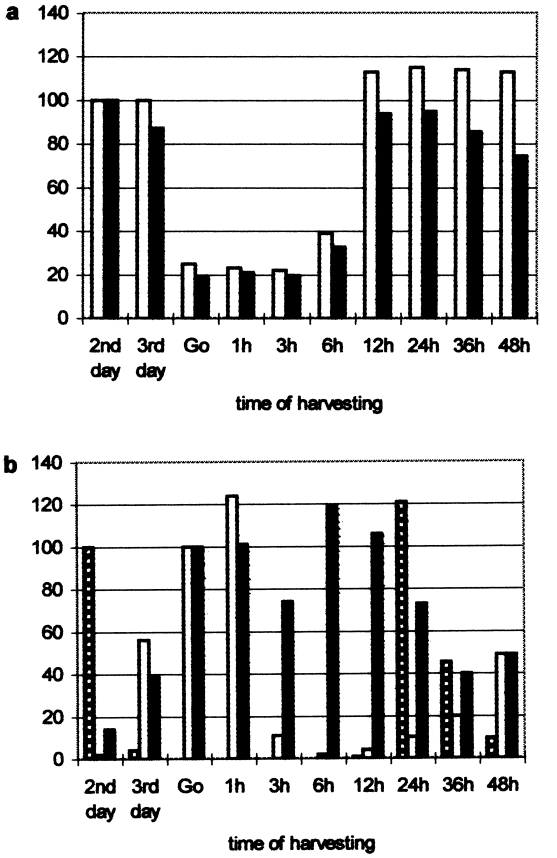

Fig. 2. a: Histograms showing the time-course of the abundance of GAPDH mRNA (white bars) in synchronized normal human dermal fibroblasts harvested as in Fig. 1. The specific mRNA radioactive signals were quantified by densitometric scanning of the autoradiograms. *Y*-axis: relative optical density. The amount of total RNA recovered from the cell cultures is also shown (black bars). The RNA recovered from the cell cultures was quantified by absorbance at 260 nm (μg of total RNA/10^6 cells). Both for GAPDH mRNA abundance and total RNA recovery data were then normalized by setting at 100% the values obtained from cells harvested on the 2nd day after plating. The data shown are representatives of four determinations obtained in separate experiments. b: Quantitation of the radioactive bands of histone H3 mRNA (check bars), clusterin mRNA (black bars) and h-gas1 mRNA (white bars). For H3, data have been normalized by setting at 100% the value obtained from cells harvested on the 2nd day after plating, while for h-gas1 and clusterin the values obtained after the induction of the quiescence state (G_0) were used. The data shown are representatives of four determinations obtained in separate experiments.

3. Results

The experimental model used in the present study was as previously detailed [18]. At 2 and 3 days after plating, at 14 days after serum starvation (quiescence state, 0 h) and at various time intervals between 1 h and 48 h following serum re-addition, the percentages of total cell population in G_0/G_1, S and G_2/M phases were detected by flow cytometry after BrdU incorporation and anti-BrdU/FITC staining. The highest percentage of cells in S phase was found 24 h after serum re-addition, while G_2/M phases peaked at 36 h. The timing of

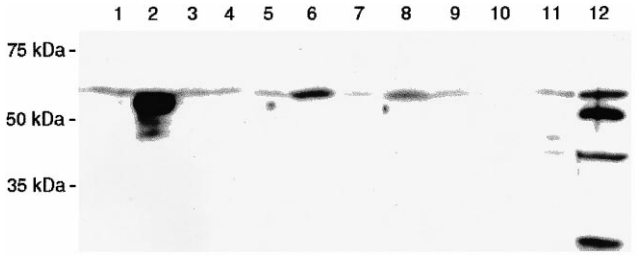

Fig. 3. Western blot analysis of clusterin protein in synchronized human dermal fibroblasts harvested as follows: lane 1, 3rd day after plating (cells at confluence); lane 2, 14 days after serum starvation (G_0, quiescence state). From lane 3 to lane 10: 3 h, 6 h, 9 h, 12 h, 18 h, 24 h, 36 h and 48 h after serum stimulation, respectively. Total protein content extracted from 4×10^5 cells was loaded on each lane and resolved by electrophoresis on 10% polyacrylamide gel. For comparison, total protein extracts from normal rat ventral prostate (lane 11) and from the ventral prostate of 4 day castrated rats (lane 12) are also shown. Clusterin immunoreactive bands were detected by chemiluminescence using polyclonal anti-rat SGP-2 antibodies. The data shown are representatives of four determinations obtained in separate experiments.

the cell cycle phases was also confirmed by assessing, by Northern hybridization of total RNA, the abundance of the transcripts coding for h-gas1, a marker of G_0 phase [20], histone H3, a marker of the S phase [21] and GAPDH, a housekeeping enzyme (Figs. 1 and 2). GAPDH mRNA exhibited a marked decrease at G_0, followed by a gradual increase from 3 h up to 12 h, and remained constant afterwards. This pattern paralleled that exhibited by total RNA recovered from cell cultures (μg/10^6 cells) under the same conditions, suggesting that changes in GAPDH mRNA accumulation are the consequence of the expected changes in the general transcriptional activity of the cells (Fig. 2a). Accumulation of the transcript coding for histone H3 (Fig. 2b) dropped dramatically in cells at confluence (third day after plating), remained undetectable until 12 h after serum re-addition, reached its highest value at 24 h (S phase) and returned to a very low level at 48 h. The h-gas1 mRNA (Fig. 2b) increased markedly on the third day after plating, continued to increase during serum starvation (G_0 phase) and up to 1 h after serum re-addition. It decreased steeply until 6 h, and started again to rise slowly up to 48 h, when it returned to the level of the third day after plating as a possible result of the proliferation activity leading cells again to confluence.

The pattern of accumulation of clusterin mRNA showed a marked, progressive increase in cells proceeding to confluence and induced to quiescence by serum starvation (from the second day after plating to G_0, Fig. 2b). One h after serum re-addition, the level of clusterin mRNA was still high, it decreased at 3 h, exhibited a second peak at 6 h and started to decline gradually at 12 h. At 48 h it was at about the same level as shown on the third day after plating.

Changes in the amount of the protein during the cell cycle phases were assessed by immunoblotting. Fig. 3 shows that a high molecular weight form of clusterin, of about 60 kDa, that is recognized by the polyclonal anti-rat clusterin antibodies we have used, markedly accumulated in human dermal fibroblasts at quiescence (G_0), together with two minor species of about 50 and 45 kDa. This is consistent with the peak of clusterin mRNA detected at G_0 phase, but a direct correlation between the protein levels and the abundance of mRNA (Figs. 2b and 3) could hardly be found at later time intervals. The correspondence of the major band with a clusterin isoform was confirmed by the fact that it appeared strongly induced in the prostate of castrated rats (Fig. 3).

4. Discussion

The data presented here, show that clusterin is a cell cycle related gene in human dermal fibroblasts, synchronized by 'serum starvation'.

The enhanced accumulation of clusterin mRNA shown by the quiescent cells can hardly be a consequence of apoptotic processes. In fact, when measuring the amount of apoptotic cells as subdiploid peak by flow cytometry, the average values found under our conditions were 0.78% of total cell population during the non-synchronous stage (2nd and 3rd day after plating), 1.30% in the quiescent state, and 0.80% at the subsequent time intervals after serum stimulation.

In cells reaching quiescence, immunoblotting experiments with anti-rat clusterin antibodies revealed a large increase in the accumulation of a protein of about 55–60 kDa and the appearance of two minor bands of about 50 and 45 kDa. The major band corresponded to one of the forms of rat clusterin that is induced in the rat prostate by androgen withdraw (Fig. 3). Increased accumulation of these clusterin isoforms matched with the increase in the abundance of clusterin mRNA occurring under the same conditions (Fig. 2b). However, the correspondence between mRNA and protein accumulation ceased after serum re-addition; in fact, the second peak of mRNA (6–12 h) was not accompanied by correspondent changes in the levels of protein (compare Figs. 2a and 3). This may find an explanation either in the possible existence of post-transcriptional regulatory events that might prevent immediate translation of clusterin mRNA during specific phases of the cell cycle, or in the inability of the antiserum used to recognize specific isoforms of clusterin that might be produced, during the different phases of the cell cycle, by intracellular differential processing [22] and/or differential splicing of clusterin mRNA [22,23], an issue which is under current investigation in our laboratory.

Support to the hypothesis that clusterin overexpression may be one of the factors leading cells to quiescence comes indirectly from our previous data. In the rat ventral prostate clusterin mRNA is undetectable in the tall columnar cell population, active in cell division or secretory function, comprising the vast majority of epithelial cells, but it is expressed in a minor population of non-dividing cuboidal cells [4], that are considered to derive from the atrophy of the columnar ones. We have shown that the relative number of these cells increases markedly with ageing [5], thus explaining the marked progressive increase in the abundance of clusterin mRNA that occurs under the same conditions [6]. However, since no increase in the rate of apoptosis was shown to accompany the progressively enhanced proportion of cuboidal cells [5], it is suggested that the latter represent a form of quiescent, long-surviving cells formed from the columnar ones, through a process in which clusterin gene relaxation may play a pivotal role. Pertinent to this may be the recent finding that clusterin is a senescence induced gene in mammalian cells [14].

On the other hand, human lymphocytes stimulated to proliferation by PHA exhibited a progressive decrease in the level of clusterin mRNA [16].

The recent report [15] that the castration induced forms of the rat prostate clusterin, including a novel species not detected in intact animals, specifically accumulate in the epithelial cells undergoing apoptosis, does not necessarily contradict this interpretation. It is in fact conceivable that, under the prevailing conditions regarding levels of trophic factors, hormones or other stimuli (including those that accumulate with ageing), specific clusterin forms might be produced that may cause the cells to follow totally different fates. Apoptotic doom or induction to quiescence are the pathways suggested by the available data. The latter alternative might lead to an atrophic state that could prevent both cell death and pathological proliferation.

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