Modulation of different clusterin isoforms in human colon tumorigenesis

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Clusterin is a ubiquitous secretory heterodimeric disulfide-linked glycoprotein, which is implicated in several physiological processes, including immune regulation, cell adhesion and morphological transformation, lipid transportation, tissue remodelling, membrane recycling and cell–cell interactions. A large number of studies have focused their interest on clusterin gene products as mediators of cell cycle progression and cell death induction, although data on the different isoforms and their role in the different cell processes are still obscure. Recently, an increased clusterin expression in breast cancer has been reported. In order to elucidate the role of clusterin in tumor progression and whether one of its isoforms is preferentially expressed in tumorigenesis, we examined its presence throughout the different steps of human colon carcinoma, one of the best-characterized models of human tumor progression. The immunohistochemical observation of 30 bioptic and surgical colon specimens demonstrated a cell compartment clusterin translocation from the nucleus to the cytoplasm directly related to tumor progression. In fact, a nuclear localization found in healthy colonic mucosa is consistent with the involvement of the proapoptotic nuclear form in the regulation of cell cycle progression and in cell death induction. The progression towards high-grade and metastatic carcinoma leads to cytoplasmic clusterin distribution. Protein extracts from freshly isolated cells of the same patients confirm in high-grade carcinomas with metastatic nodes the complete loss of the proapoptotic nuclear form and a cytoplasmic overexpression of the highly glycosylated form. Data obtained from *in vitro* experiments confirm that this form is released in the extracellular space and corresponded to the fully glycosylated one. These data suggest that the controversial data on clusterin function in tumors may be related to the pattern shift of its isoform production. As the secreted form of clusterin is correlated to cell matrix formation, cell membrane remodeling and cell–cell adhesion, the overexpression of this form in highly aggressive tumors and metastatic nodes could be a potential new prognostic and predictive marker for colon carcinoma aggressiveness.

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Introduction

Clusterin/Apolipoprotein J (ApoJ) is a 76–80 kDa heterodimeric highly conserved glycoprotein being expressed in a wide variety of tissues and found in all human fluids. It has been demonstrated that clusterin is implicated in several diverse physiological processes such as sperm maturation, lipid transportation, complement inhibition, tissue remodeling, membrane recycling, cell–cell and cell–substratum interactions, stabilization of stressed proteins in a folding-competent state and promotion or inhibition of apoptosis (Murphy *et al.*, 1988; Aronow *et al.*, 1993; Fratelli *et al.*, 1996; Ho *et al.*, 1998; Humphreys *et al.*, 1999; Humphreys *et al.*, 1999; O'Sullivan *et al.*, 2003).

Although some data have demonstrated its expression in relation to tumorigenesis suppression (Kyprianou *et al.*, 1991) and to proliferative regulation of prostate cancer cells (Bettuzzi *et al.*, 2002; Zhou *et al.*, 2002), its role in tumorigenesis is still unclear. Paradoxically, its overexpression in breast carcinomas suggests that clusterin expression increases in relation to tumor progression (Redondo *et al.*, 2000). The human clusterin comprises 449 amino acids, generating an unglycosylated holoprotein (precursor form, pCLU) with a predicted molecular mass of 60 kDa that could be proteolytically cleaved into α and β subunits held together by disulfide bonds. Mature clusterin is glycosylated and secreted as a protein of 76–80 kDa (Burkey *et al.*, 1991; Wong *et al.*, 1993; Lakins *et al.*, 1998), depending on the degree of glycosylation, which appears like a 40 kDa α and β protein smear by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions (Leskov *et al.*, 2003).

Recently, was demonstrated the presence of two different mRNA transcripts for clusterin, derived from an alternative splicing, one coding for the secreted form of clusterin and the other coding for the 50–55 kDa nuclear form without leader peptide (Konstantin *et al.*, 2003). The role of nuclear proapoptotic clusterin form is

still unclear, although it was demonstrated that its activation is related to cell cycle arrest and cell death induction. Recently, it has been demonstrated that the clusterin nuclear form (proapoptotic form) is unglycosylated and it is involved in apoptosis induction (Leskov et al., 2003). Data on the preferential induction of the proapoptotic clusterin form after irradiation suggest that the transcription of one of the two mRNA forms is closely linked to the cellular state of the cell and could be influenced by the intracellular or extracellular milieu such as cytokines, growth factors and stress-inducing agents (Reddy et al., 1996; Yang et al., 1999; O'Sullivan et al., 2003). Moreover, confocal microscopy experiments revealed an apparently inactive nuclear clusterin form in the cytoplasm of nonirradiated cells (Yang et al., 2000; Konstantin et al., 2003) that translocate to nuclear regions after ionizing radiation colocalizing with nuclear Ku70/86 heterodimer involved in apoptosis induction and DNA repair (Yang et al., 1999, 2000).

Here, we discuss the role of clusterin/ApoJ expression in the colon adenoma–carcinoma sequence that represents one of the best studied and characterized models of tumor progression. Therefore, we have investigated whether changes in its expression and cellular localization could be related to tumor progression raising the possibility that the overexpression of one specific isoform, during cellular neoplastic transformation, might cause a predisposition to cancer metastasis and tumor aggressiveness. We assess the pattern shift of clusterin isoforms in relation to the proliferative state of the cell through ex vivo and in vitro experiments in human tumor cells from biopsies and in a human colon carcinoma cell line (Caco) treated with a cytostatic compound (SST). Moreover, a correlation between the presence of a particular clusterin isoform and tumor progression has been demonstrated.

This report provides a novel link among the expression of the secreted glycosylate clusterin isoform, the disappearance of proapoptotic nuclear clusterin form and tumor progression, explaining at least in part the conflicting data on clusterin function in cell proliferation and apoptosis induction.

Results

Clusterin overexpression in colon carcinoma progression

Immunohistochemistry was performed to verify whether clusterin was expressed in colon tissue and if its expression was related to neoplastic transformation from healthy tissue to adenoma and carcinoma. Colon carcinomas were characterized by grading and staging according to WHO and TNM classifications. Table 1 summarizes the main clinical and histological features correlated to clusterin expression evaluated by immunohistochemistry as described in Materials and methods.

Clusterin expression – as shown in Figure 1 – was related to the tumor stage and grade. Clusterin expression in the colonic mucosa of healthy subjects

Table 1 Clusterin expression in the adenoma–carcinoma sequence; main clinical and histological features correlated to clusterin expression evaluated by immunohistochemistry as described in Materials and methods

Number of cases	Diagnosis	Clusterin expression	
		Nucleus	Cytoplasm
30	Healthy colonic mucosa	+	−
10	Adenoma	+	+
10	Adenoarcinoma (G1–G2; any T; N0)	−	+ + +
10	Adenocarcinoma (G2–G3; any T; N1,N2, M0, M1)	−	+ + + +

(five cases) and in the normal mucosa aside from the neoplasia (30 cases) was related to nuclear compartment, whereas in the cytoplasm the staining was almost negative as shown in Figure 1 (panel A). Colonic adenomas presented positive staining both in the nuclei and in the cytoplasm and its expression was increased as compared to normal mucosa as summarized in Table 1. Low-grade carcinomas displayed an increased clusterin expression only in the cytoplasm, whereas no positive staining was detected in the nucleus. As shown in Figure 1, clusterin expression strongly increased in carcinomas (G1–G2;pT2–T3N0M0). Moreover, cytoplasmic clusterin was found to be overexpressed in highly aggressive and metastatic tumors (G2;pT4N1M0/ M1), whereas the presence of nuclear clusterin was completely lost. As shown in Figure 1 (panel C), no antibody staining was visible in the nuclei lightly counterstained with hematoxylin. The immunohistochemical observation of highly aggressive and metastatic tumors (G2;pT4N1M0/M1) show in these tumors that clusterin could also be released in the extracellular space (Figure 1, panel C).

A TUNEL assay performed on the same sections (data not shown) demonstrated that the apoptotic index (apoptotic cells number: total cells number) was inversely related to the increased cytoplasmic clusterin expression and to the grade and stage of the tumors.

Overexpression of the clusterin secreted form is directly correlated with tumor progression

Protein extracts were prepared from tumor biopsies to assess which isoform was overexpressed in tumor tissues as compared to their normal counterpart.

Four tumor biopsies were analysed; two out of four were carcinomas without nodal involvement (G2;pT3N0M0) and the other two were metastatic tumors (G2;pT4N1M0/M1). Western blot analysis was performed to evaluate clusterin expression, and protein extracts from normal tissues of the same patients were used as control. The results obtained with clusterin MoAb anti-alpha chain (Figure 2), described in Materials and methods, demonstrated the presence of different clusterin isoforms. Different clusterin isoforms have been recently characterized and the antibody used was able to detect all the isoforms as described in Materials and methods.
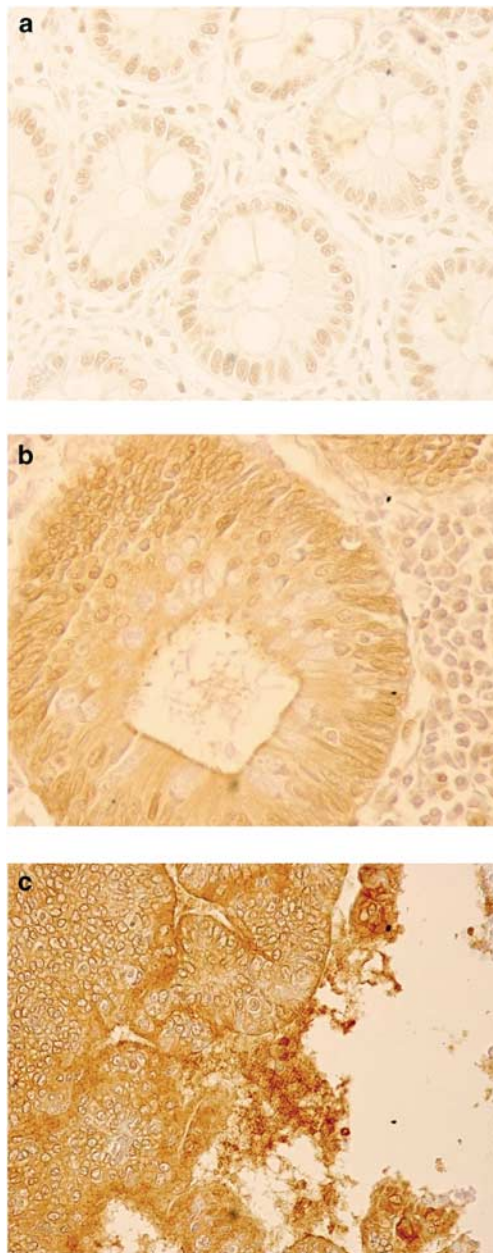
Figure 1 Immunohistochemical analysis of clusterin expression in human colon cancer progression: (**a**) normal mucosa without atypias – nuclear clusterin expression; (**b**) adenoma – nuclear and cytoplasmic clusterin localization; and (**c**) invasive colon carcinomas. Clusterin is overexpressed and localized only in the cytoplasm. All the sections were light counterstained with hematoxylin to avoid interference with the nuclear-specific DAB staining. Magnification × 60
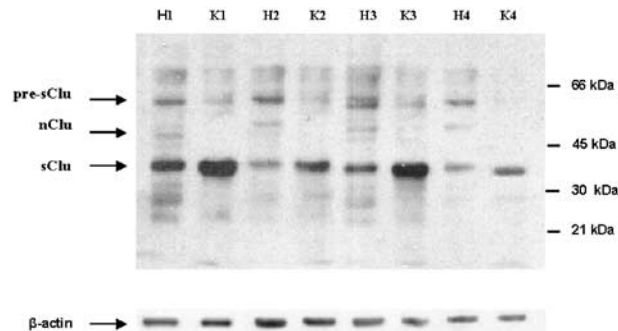

Figure 2 Western analysis of clusterin expression in tumor (K) and healthy (H) tissues. Representative results from three independent experiments are shown. Protein extracts were prepared from tumor and healthy tissues of the same patient used as internal control. (H1–4) Clusterin expression in normal tissues of different patients. (K1–4) Protein extracts from tumoral tissues from the same patients: (K1) highly infiltrative carcinoma with positive nodes and liver metastasis; (K2) nonmetastatic colon carcinoma, (N0, M0); (K3) invasive carcinoma with positive nodes; (K4) nonmetastatic colon carcinoma, (N0, M0). sCLU, secreted α-subunit of the mature protein (40 kDa); nCLU, nuclear apoptosis-associated clusterin (50–55 kDa); and pre-sCLU, uncleaved and precursor form of the secreted clusterin (60 kDa). Overexpression of the 40 kDa (sCLU) form in highly infiltrative metastatic carcinomas (K1 and K3) and disappearance of the proapoptotic-nuclear (nCLU, 50 kDa) form in tumors (K1–K4)

tissues, even though in the latter group this form was overexpressed (2–4 times) (Figure 2). As shown in Figure 2, a 1.5–4-fold increase of clusterin secreted form (40 kDa) was observed in all the analysed tumors as compared to their normal counterpart. In the two highly aggressive and metastatic tumors (G2pT4N1M0/M1), the secretory form was found to be four times increased as compared to the normal counterpart. It is worth noting that the normal tissue of these samples also displayed a strong increase of the clusterin 40 kDa form as compared to other normal counterparts.

The translocation of the clusterin 50 kDa isoform from the cytoplasm to the nucleus is modulated by a cytostatic and proapoptotic treatment

In vitro experiments were performed to determine whether the expression of the nuclear proapoptotic clusterin form, completely lost in highly aggressive tumors, was involved in apoptosis induction. A colon carcinoma cell line, Caco was treated at a different time with a cytostatic hormone, somatostatin. The presence of different clusterin forms was evaluated in the nuclear and cytoplasmic cell compartment.

Figure 3 shows the Western blot analysis of clusterin expression in cytoplasmic (panel A) and nuclear protein (panel B) extracts from untreated, 4 and 24 h SST-treated cells. SST specifically modulates the proapoptotic clusterin form (50 kDa) in the different cell compartments. A strong decrease in the clusterin proapoptotic form was observed within the cytoplasm after 4 h of SST treatment with a concomitant twofold increase in this form within the nucleus. After 24 h of SST treatment, an increased clusterin expression can be observed, affecting especially the 50 kDa proapoptotic

Clusterin precursor holoprotein form (60 kDa) was present both in normal and tumoral tissues, although the level of this form was two times higher in the former than in the latter. The expression of the proapoptotic nuclear form (50 kDa) was evident in normal mucosa conversely; this form was completely lost in all the tumoral tissues (four out of four samples, G2pT3N0M0, G2pT4N1M0/M1). The clusterin form of 40 kDa corresponding to the secreted form (alpha chain 40 kDa) was present both in normal and in tumoral
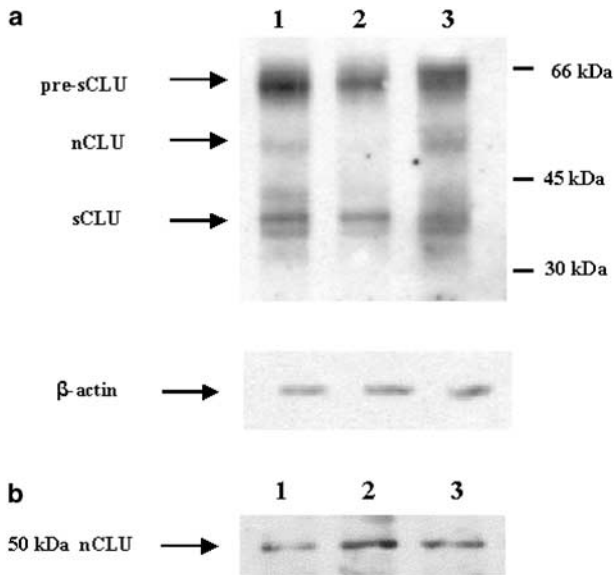
Figure 3 Representative Western blot analyses of different pattern shifts of clusterin forms after different SST treatment times in human carcinoma cell line (Caco-2). (**a**) Cytoplasmic extracts from untreated cells (line 1), 4 h (line 2) and 24 h (line 3) treated cells. (**b**) Nuclear extracts at the same treatment time. Untreated (line 1), 4 h (line 2) and 24 h (line 3) treatment

form. To confirm clusterin isoforms' modulation following antiproliferative treatment, we performed an *ex vivo* experiment on freshly isolated colon cells from a human low-grade carcinoma (G1/G2N0M0) and healthy colonic mucosa from the same patient. The 40 kDa secreted form that was present in the normal mucosa was 4.5 times (as compared to the normal counterpart) over-expressed in tumor cells, as shown in Figure 4. After 24 h, SST treatment induced a twofold decrease in the 40 kDa form, whereas the nuclear proapoptotic form was 1.5 times increased as compared to the untreated tumor cells in the *ex vivo* isolated tumor cells. These data fully confirmed a modulation of the different isoforms after SST treatment to Caco cell line.

Characterization of the clusterin isoforms

To establish whether the different clusterin species identified in tumor and healthy extracts, corresponded
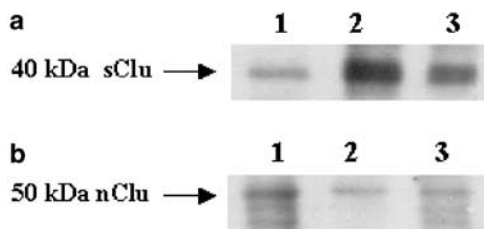

Figure 4 Panel a: Modulation of the clusterin secreted form (40 kDa) forms in whole-cell lysate from freshly isolated normal colonic mucosa cells (sample 1) and from untreated tumor cells (lane 2) and 24 h SST-treated tumor cells (lane 3) of the same patient. Panel b: Western blot analysis of the proapoptotic form: expression in normal colonic cells (lane 1), untreated tumor cells (lane 2) and 24 h SST-treated tumor cells (lane 3)

to different forms of the protein or to different stages of glycosylation of the same protein, cytoplasmic and nuclear protein extracts from Caco cells were incubated with a deglycosidase as described in Materials and Methods. The assay of deglycosylation permitted to identify the glycosylation status of all the different clusterin isoforms. All the species that were glycosylated after the enzyme treatment, loaded in an SDS denaturing polyacrylamide gel, shifted their molecular weight after the *in vitro* incubation in the presence of the deglycosylase PNGaseF. Control reactions included protein processed identically but in the absence of the enzyme. As shown in Figure 5 in the cytoplasmic extracts, the form with a molecular mass of 60 kDa corresponded to the uncleaved and unglycosylated α–β precursor protein because the holoprotein (precursor form) did not alter its electrophoretic mobility after treatment. Size analysis of Western immunoblot demonstrated that the 50 kDa nuclear proapoptotic form of clusterin also is not glycosylated because treatment with PNGase F did not alter the electrophoretic mobility of the protein (Figure 5, lane B), demonstrating the absence of any N-linked oligosaccharides. Conversely, the 42 kDa band corresponded to the glycosylated α-subunit of mature secreted protein; in fact, after incubation with PNGase F (Figure 5, lane B) it shifted the mobility from 40 to 28 kDa.

In order to better clarify that the nuclear 50 kDa form also was not glycosylated, we performed the same deglycosylation assay, described in Materials and methods, also on nuclear extracts from Caco cells. As
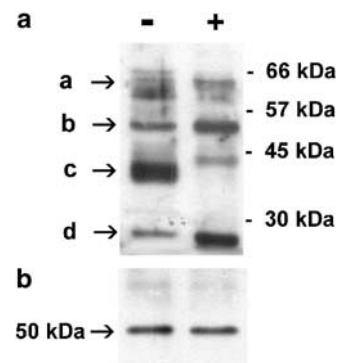

Figure 5 Characterization of glycosylated isoforms after deglycosylation treatment. Representative results from three replicate experiments are shown. Panel a: Glycosylation state of the cytoplasmic isoforms of clusterin. Purified cytoplasmic proteins of Caco-2 cells were treated with (+) or without (−) 1000 U PNGase F as described in Materials and methods and analysed by Western blot. (**a**) 60 kDa unglycosylated, uncleaved secreted clusterin precursor protein; (**b**), 50 kDa unglycosylated nuclear clusterin; (**c**), 40 kDa glycosylated cleaved α-chain (secreted clusterin); (**d**) 28 kDa deglycosylated cleaved α-chain. Panel b: Glycosylation state of nuclear clusterin isoform (panel B). Proteins were treated with (+, on the right) or without (−, on the left) the PNGase F treatment that abrogate protein glycosylation. Purified nuclear samples of Caco-2 cells after 4 h of SST treatment were treated with (line +) or without (line −) 1000 U PNGase F; no shifted bands were detected, demonstrating that this nuclear clusterin form is not glycosylated. Samples were normalized for equal loading as described in Materials and methods
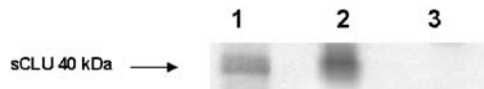
Figure 6 Western immunoassay of TCA immunoprecipitation of cell culture medium. Lane 1: Whole-cell lysate; the intracellular cytoplasmic 40 kDa form; lane 2: the 40 kDa form present in cell's culture medium corresponding to the glycosylated form; lane 3: complete medium without cell incubation used as internal control, no clusterin present in the medium

Caco cells have a relatively poor quantity of nuclear proapoptotic form, we obtained an increase of the nuclear form on treating the colon carcinoma cell line for 4 h with SST. The nuclear extracts were then treated as described above with the deglycosidase PNGase F and analysed by Western blot. As shown in Figure 5, the nuclear proapoptotic form of clusterin isolated from nuclear protein extracts of Caco cells after 4 h of treatment with SST did not alter its electrophoretic mobility after PNGase as compared to the untreated control. This result demonstrated that the nuclear form of 50 kDa corresponded to an unglycosylated isoform.

Tumor-overexpressed clusterin form is secreted

To assess whether the clusterin glycosylated form (α-subunit 40 kDa) found to be upregulated in neoplastic cells cytoplasm was extracellularly released and corresponded to the secretory form, a TCA precipitation was performed. The culture medium of untreated Caco-2 cell line cultured for 7 days was TCA precipitated. Precipitated proteins were analysed for the presence of different clusterin forms in the medium by Western blot analysis. Precipitated proteins from medium alone were used as control. The whole-cell lysate from untreated cells was loaded on the same polyacrylamide gel to compare the molecular weight and the intracellular protein pattern to the secreted form. Figure 6 demonstrated that only the fully glycosylated clusterin form (40 kDa) was detected by the MoAb against the clusterin alpha chain subunit, in the culture supernatant. This protein corresponded to the intracellular 40 kDa present in the whole-cell lysate from untreated Caco-2 as shown in Figure 6. These data demonstrated that the glycosylated clusterin overexpressed in Caco cell line and in highly aggressive tumors corresponded to the secretory form and it is released in the extracellular space, confirming the observation of the immunohistochemistry of highly aggressive tumor tissues shown in Figure 1 (panel C).

Discussion

In this report, we provide the evidence that clusterin/ApoJ is overexpressed in highly aggressive colon tumors and metastatic nodes. Moreover, its cellular localization, shifting from the nucleus, in healthy colonic mucosa, to the cytoplasm in tumors, seems to be directly related to the stage of the disease. Despite the original hypothesis that clusterin is a marker for programmed cell death (French *et al.*, 1992), several experiments and clinical studies have demonstrated conflicting findings on its role in tumors (Redondo *et al.*, 2000; Miyake *et al.*, 2001; Zellweger *et al.*, 2001; Zhou *et al.*, 2002). Experimental results obtained in SCID mice injected with clusterin-transfected human renal carcinoma cells indicate that clusterin overexpression may contribute both to enhance cancer cell survival, preventing apoptosis, and to increase the metastatic potential. Moreover, *in vitro* studies showed that clusterin overexpression stimulates cell motility and invasive ability in human renal cell line (Miyake *et al.*, 2001). Collectively, these findings suggest that clusterin upregulation plays a protective role against apoptosis induced by various kinds of stimuli, and thereby may confer an aggressive phenotype during cancer progression (Redondo *et al.*, 2000; Miyake *et al.*, 2001). The observation on clusterin expression throughout the different steps of colon carcinoma progression demonstrated the presence of the nuclear form in the nuclei of the normal mucosa. As the nuclear form has been demonstrated to be involved in cell cycle regulation and apoptosis induction, this result suggests that in a normal cell-proliferative state of the colonic mucosa, this protein could be probably involved in cell cycle regulation and apoptosis induction. It has been proved that only the unglycosylated clusterin form can cross the nuclear membrane; therefore it seems probable that this form may be involved in apoptosis induction. In fact, it has been demonstrated that after irradiation only this form could inhibit Ku70/86 activation interacting with Ku70 subunits, preventing the DNA double-strand breaks repair and inducing cell death (Yang *et al.*, 1999; Pucci *et al.*, 2001; Rigas *et al.*, 2001; Leskov *et al.*, 2003).The overexpression of the clusterin secreted form, found only in the cytoplasm of highly infiltrating tumors and metastatic nodes, is due to a pattern shift of cancer cells clusterin forms. In fact, the increased level of the secreted form and the disappearance of the nuclear unglycosylated one are directly connected to increased cell survival, aggressiveness and enhanced metastatic potential. The *in vitro* study in a colon carcinoma cell line treated with a cytostatic compound and apoptosis inducer demonstrated a modulation of the different isoforms and the redistribution of the proapoptotic form from the cytoplasm into the nucleus only after 4 h of treatment and an increased production after 24 h of treatment. The 24 h of SST treatment induces even in freshly isolated cells from colon adenocarcinoma an increase of the proapoptotic form (1.5 times) and at the same time strongly reduces (two times) the formation of 40 kDa form, overexpressed in highly aggressive tumors, whose correspondence to the secreted form we also demonstrated. Data on the glycosylation status of the different clusterin isoforms confirmed that the nuclear form of 50 kDa absent in tumoral tissues was not a glycosylated form. These results suggest that the nuclear unglycosylated clusterin form, resulting from the translation of an alternative spliced RNA as previously demonstrated (Leskov *et al.*, 2003), is directly involved in cell cycle regulation and apoptosis induction. The

inverse correlation concerning clusterin isoforms expression found in tumors may support the hypothesis that protection from the apoptosis is due almost in part to the disappearance of the nuclear form in tumors or to the inhibition of the translocation in the nucleus of this form. In fact, the enhanced tumor cell survival is correlated to the loss of the proapoptotic form and with the overexpression of the secreted form that could be involved in the increased invasive ability and motility, and probably in conferring an enhanced metastatic potential. In our *in vitro* system, the SST treatment induces an increase in the clusterin nuclear form that is directly related to apoptosis induction. By virtue of the previously published data that demonstrated the cytostatic and proapoptotic action of somatostatin hormone, the restoration of the nuclear proapoptotic form both in the colon carcinoma cell line and in the cells isolated from a human tumor indirectly demonstrated the involvement of this form in the regulation of cell proliferation and apoptosis induction. Moreover, the cytostatic treatment reduced the 40 kDa form overexpressed in metastatic tumors. These results suggest that the expression and the subcellular localization of one specific isoform could be modulated by external growth-promoting or -inhibiting factors.

The findings in the current study point out a possible role of clusterin in colon tumorigenesis and in the disease progression. Thus, these results demonstrated the correlation between the enhanced cell survival and the disappearance of the proapoptotic form in aggressive tumors with a concomitant increased production of the secreted form. These data on clusterin isoforms pattern shift could almost in part clarify the conflicting data upon the presence of clusterin and apoptosis inhibition or induction. More exhaustive studies are needed to rule out the value of clusterin expression as a new survival prognostic factor. Currently, extensive studies on secreted clusterin level are actively investigating whether the release of the soluble form in the serum could be considered a new prognostic circulating marker and a potential new therapeutic target.

Materials and methods

Immunohistochemical staining and TUNEL assay

Sections, 5-μm thick from each tumor, and the normal counterpart were mounted on frozen plus slides, deparaffinized and rehydrated through xylene and alcohol. After cooling for 15 min in jars containing 10 mmol/l citrate buffer, endogenous peroxidase was blocked in 3% H_2O_2 in 0.1% sodium azide for 15 min. The detection of clusterin was obtained using a commercial monoclonal primary antibody able to detect all the isoforms. Clusterin monoclonal antibody anti-α chain was obtained by Upstate lotto 18736 clone 41D. The specificity was demonstrated in the original publication of Bandyk *et al.* (1990) and its specificity was compared by using also the commercial polyclonal antibody Santa Cruz (rabbit polyclonal antibody H330, Santa Cruz Biotechnologies, Inc.), recently published to recognize all the clusterin isoforms, obtaining the same results. The clusterin monoclonal antibody (Upstate Cat. 05–354) was used at 1:400 dilution for overnight incubation at 4°C. After TBS washes, the sections were incubated with biotinylated

goat anti-mouse IgG (1:40; Ylem), and then with peroxidase-labeled streptavidin. The staining was completed after 3 min of incubation with a freshly prepared substrate–chromogen solution. Sections were washed in running tap water and lightly counterstained with hematoxylin followed by dehydration and coverslip mounting. Negative controls were obtained by omitting the primary antibody developed using diaminobenzidine tetrahydrochloride as chromogen. Clusterin expression was scored as follows: negative if no staining was seen or if immunoreactivity was observed in less than 10% of tumor cells, and positive if more than 10% of the cells showed staining. All slides were blindly evaluated for immunostaining and protein localization without any knowledge of the clinical outcome and clinical data.

For TUNEL assay, *in situ* labeling of the 3'-ends of the DNA fragments generated by apoptosis-associated endonucleases was performed using the protocol of a commercial apoptosis detection kit (Boehringer Mannheim, Germany). Established morphological features used to identify apoptosis on H&E staining were also required in TUNEL-stained slides. A total of 1000 cells were counted for each specimen.

Cell culture

Colon carcinoma cell lines Caco-2 (American Type Culture Collection, ATCC) were seeded at subconfluent density (6×10^4 cells/cm^2). At 1 day after seeding, cells were treated with somatostatin at a final concentration of 10^{-5} M. Untreated cells were used as control. Treated and untreated cells were seeded in triplicate.

Preparation of cytoplasmic and nuclear extracts

Treated and untreated cells were processed for protein extraction. Whole-cell extracts were obtained by tissue homogenizations. Cells were lysed in ice-cold 1% Triton lysis solution (50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glucose, 1% Triton X-100, 0.1% SDS, 15 mM MgCl$_2$, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin) for 30 min on ice. Insoluble matter was removed by centrifugation, and the protein concentration was measured by Bradford (Bio-Rad, Richmond, CA, USA) assays. Postnuclear and nuclear extractions were performed according to Dignam (Dignam JD, 1983; Andrews NC *et al.*, 1991). Cells were washed twice with PBS and centrifuged at 1100 r.p.m. for 5 min. The pellet was suspended in 0.2 ml ice-cold buffer A (HEPES–KOH 10 mM, pH 7.9, MgCl$_2$ 1.5 mM, KCl 10 mM, EDTA 1 mM); cells were allowed to swell on ice for 10 min. The homogenate was centrifuged for 1 min in a microfuge at 10 000 r.p.m.; the postnuclear extract containing cytoplasmic proteins was carefully removed from the nuclear pellet and stored at −80°C.

The nuclear pellet was resuspended in ice-cold NaCl-extraction buffer and incubated on ice for 15 min. Cellular debris was removed by centrifugation in a microfuge at 10 000 r.p.m. for 5 min. The supernatant fraction, containing DNA-binding proteins, was stored at −80°C. All protein extraction steps were checked by means of an optical microscope.

Protein content in the cytoplasmic and nuclear extracts was determined in triplicate by Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories-Munchen). Bovine serum albumin was used as standard.

Western blotting

Nuclear, cytoplasmic and whole-protein extracts (15 μg) were denatured under reducing conditions (1.0% β-mercaptoethanol) in sample buffer and separated by 10% SDS–PAGE.

Proteins were transferred onto a PVDF membrane (Hybond-P, Amersham-LIFESCIENCE) using an electroblotting apparatus. Membranes were stained with Poinceau S dye, to check equal loading and homogeneous transfer. Nuclear, cytoplasmic and whole-protein extracts (15 μg) were denatured under reducing conditions (1.0% β-mercaptoethanol) in sample buffer and separated by 10% SDS–PAGE. A molecular weight marker was loaded on the same gel (Amersham-LIFESCIENCE). Proteins were transferred onto a PVDF membrane (Hybond-P, Amersham-LIFESCIENCE) using an electroblotting apparatus. Its specificity in Western blot was compared to the commercial polyclonal antibody Santa Cruz (rabbit polyclonal antibody H330, Santa Cruz Biotechnologies, Inc.), recently published to recognize all the clusterin isoforms, obtaining the same results. After incubation, the membranes were washed extensively and then incubated with 1 : 5000-diluted horseradish peroxidase conjugated secondary antibody (anti-mouse IgG, Santa Cruz Biotechnologies, Inc.) for 1 h at room temperature. Filters were reprobed with anti-β-actin (Sigma-Aldrich, Saint Louis, MO, USA) or anti-Sp-1 (Santa Cruz Biotechnologies, Inc.) mouse IgG1 monoclonal antibodies, to normalize cytoplasmic and nuclear protein levels, respectively. Filters were washed and developed using an enhanced chemiluminescence system (ECL Amersham-Pharmacia Biotech). The optical densities were obtained by scanning densitometry, after normalization for nuclear or cytoplasmic housekeeping gene products Sp-1 and β-actin; all the experiments were repeated three times and gave similar results.

Deglycosylation of N-linked oligosaccharides assay: purified nuclear and cytoplasmic proteins (15 μg) of Caco cell line were denatured in 0.5% SDS, 1% β-mercaptoethanol for 10 min at 100°C and incubated overnight at 37°C with 0.5 M sodium phosphate buffer and 10% NP-40, pH 7.5 and 1000 U of PNGase F (NEB) in a total volume of 30 μl. Control reactions included proteins from the two compartment treated with the same conditions but in the absence of the enzyme. Protein obtained from the two subcellular compartments, cytoplasm and nucleus, after incubation with the deglycosidase, having no glycosylated residues, were loaded in a polyacrylamide denaturing gel as described above. Proteins from the same compartment that had not been treated with the enzyme were used as control. Nuclear, cytoplasmic protein extracts (15 μg) were denatured under reducing conditions (1.0% β-mercaptoethanol) in sample buffer and separated by 10% SDS–PAGE. Proteins were transferred onto a PVDF membrane (Hybond-P, Amersham-LIFESCIENCE) using an electroblotting apparatus. A molecular weight marker was loaded in the same gel to determine the molecular weight shift after the deglycosylation assay. The detection for the presence of all the clusterin forms with or without the deglycosidase treatment was performed as described above.

Western blot analysis of clusterin released in culture medium

Caco cells were seeded at a density of 7×10^4 cells/cm^2 in 75-cm^2 flasks containing DMEM supplemented with 10% FBS and nonessential amino acids and incubated until the culture reached subconfluence status (approximately 7 days). Then, 1 ml of culture medium was reserved and centrifuged, and the supernatant fraction was precipitated by the addition of ice-cold trichloroacetic acid at a final concentration of 15%. Precipitate was washed twice with 95% ethanol, dried and resuspended in a buffer containing 62 mM Tris-HCl, pH 6.8, 1% SDS, 15% glycerol, 0.04% bromophenol blue and 10% β-mercaptoethanol. A cell-free medium that has been used for an internal control was submitted to the same treatment. All precipitated proteins were loaded on 10% SDS–polyacrylamide gels and electroblotted onto a PVDF transfer membrane (Amersham-Pharmacia Biotech). Western analysis was performed as described above.

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