CLUSTERIN (SGP-2, ApoJ) EXPRESSION IS DOWNREGULATED IN LOW- AND HIGH-GRADE HUMAN PROSTATE CANCER

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CLUSTERIN is overexpressed during tissue and cell involution and downregulated in proliferating cells. Its role in cell survival, cell death and neoplastic transformation remains debated. We studied the expression and distribution of clusterin mRNA and protein in human prostate carcinoma (CaP) specimens of different degrees of malignancy. Fresh CaP specimens were obtained from 25 patients subjected to long-term androgen ablation before surgery. Clusterin expression was assessed by Northern and Western analysis, in situ hybridization and immunohistochemistry, in comparison with GAS1 and histone H3 mRNA (markers of cell quiescence and S phase of the cell cycle, respectively). Clusterin is downregulated in CaP in comparison with matched benign controls. In low-grade CaP, clusterin colocalized with GAS1 to the stromal compartment, and in some glands, the basal lamina was heavily stained. In high-grade CaP clusterin stained the remnants of stromal matrix while histone H3 localized to cancer cells, which were very rarely clusterin positive. High clusterin expression was found in the branches of a nerve infiltrated by tumor. The periglandular clusterin expression found in low-grade CaP could result from induction of quiescence and/or apoptosis of prostatic fibroblasts lining those glands in which tumor invasion is at an initial stage, involving basal lamina. In advanced CaP, the staining of the remnants of the extracellular matrix suggests a role for clusterin in the process of dismantling the stromal organization caused by cancer progression.

Key words: clusterin (SGP-2, ApoJ); ornithine decarboxylase (ODC); prostate cancer; in situ hybridization; immunohistochemistry; gene expression

Clusterin was originally cloned as the most potently induced transcript during rat prostate regression after castration1 and then found to be upregulated in several other experimental models of tissue involution or cell atrophy.1-5 Its level of expression was shown to depend on the proliferation state, being upregulated in quiescent human fibroblasts,6 but downregulated during cell proliferation.7 In the prostate gland from normal or aged rats, clusterin accumulated in epithelial cells of the cuboidal type, which are considered to be derived from atrophic involution of the actively secreting and proliferating columnar cells. Columnar cells represent the great majority of the prostatic epithelial cell population and are devoid of clusterin.8-9 In the regressing rat prostate after androgen ablation by orchidectomy8 or finasteride treatment,10 clusterin becomes transiently overexpressed and accumulates in the columnar cells undergoing apoptosis.

It has been found recently that the clusterin gene encodes a family of different proteins11 all derived, by alternative posttranslational processes, from the same precursor of about 53 kDa.12,13 Different biologic functions have been hypothesized for these alternative isoforms.12,13 Reports from several laboratories suggested a role for this protein in supporting cell survival.14-16 At the same time, at least one form of clusterin appeared to be involved in programmed cell death induction.17,18 In any event, it appears that alterations in clusterin expression and/or protein maturation are linked to changes in tissue growth or regression, which may be related to proapoptotic and antiapoptotic forms of clusterin.

A role of clusterin in neoplastic transformation and progression has not been yet completely clarified. Data on the antiapoptotic activity of clusterin point at a function for this protein in promoting cancer development.19 We previously found, under different experimental conditions, that clusterin mRNA is downregulated in both low-grade and high-grade cancer. This is among the earliest events in human prostate carcinoma (CaP) progression.20

In order to obtain further insight into the role of clusterin in CaP development and progression, we studied, by Northern and Western analyses, in situ hybridization and immunohistochemistry, the levels of expression and the regional and cellular distributions of clusterin mRNA and protein in CaP specimens of different degrees of malignancy.

MATERIAL AND METHODS

Patient treatments

Our study was conducted with surgical specimens from the prostate of patients affected by prostatic adenocarcinoma as previously described.20 All patients were subjected to the same standard protocol of long-term androgen ablation, i.e., androgen-suppression therapy for 3 months before surgery, according to the following standard protocol: 200 mg/day of cyproterone acetate, plus a single dose (3.75 mg) of the luteinizing hormone-releasing hormone (LHRH) agonist leuprolidine for the first month of treatment, followed by leuprolidine alone for the next 2 months. This investigation was performed after approval by the local Human Investigation Committee and after obtaining informed assent from the patients involved in our study.

Patient samples

Radical prostatectomy specimens were obtained from 25 patients bearing tumors that were graded from Gleason grade 1–5 (Gleason score 2–9) by the pathologist that assisted us in our previous work.20 Briefly, immediately after total prostatectomy, a

Abbreviations: CaP, prostate cancer; GAS1, growth arrest specific gene 1; KSFM, keratinocyte serum-free medium; ODC, ornithine decarboxylase; SGP-2, sulfated glycoprotein 2 (clusterin); SV40, simian virus 40.

Grant sponsor: AIRC 2002, Investigator Grant “Clusterin and Polyamine Regulatory Genes as New Molecular Markers for Prostate Cancer Progression and Therapy.”

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Received 14 February 2003; Revised 16 April 2003, 6 June 2003; Accepted 18 July 2003

DOI 10.1002/ijc.11496
specimen of about 0.5 cm³ in size was excised out from the cancerous portion (C) of each individual gland. A second, well-separated area of benign (normal) tissue (N) was also dissected out. Both specimens were quickly frozen on a flat glass surface, covered with dry ice powder at −80°C. By this procedure the tissue orientation could be preserved, while a suitable flat cutting surface for the cryostat was generated. Starting from the top surface, 14 µm sections and a tissue slice of about 200 mg (fresh weight) were obtained from both C and N specimens. The 14 µm tissue sections were stained and used for the histopathologic and morphologic characterization of the thick tissue slice comprised between 2 of the above sections. The thick 200 mg slice obtained from each specimen was used for Northern or Western blot analyses. The pathologist confirmed the presence of neoplastic tissue and assessed Gleason grading and scoring directly on each specimen studied. Only N specimens with no sign of benign prostatic hyperplasia (BPH) or tumor invasion and with an epithelial and stromal cell content similar to that of the C counterparts were used as controls. The trend in clusterin expression was evaluated in a panel of 25 patients: in particular, well differentiated (score 2–4), n = 5; moderately differentiated (score 5–6), n = 11; and poorly differentiated (score 7–10), n = 9.

Primary cultures of epithelial and stromal cells from CaP specimens

Primary cultures of epithelial and stromal cells were obtained from a small fragment of the same CaP specimen manually minced, placed at 37°C and shaken overnight in a 50 ml conical flask containing 10 ml RPMI-1640, 2% FCS and 200 g/ml minced, placed at 37°C and shaken overnight in a 50 ml conical flask containing 10 ml RPMI-1640, 2% FCS and 200 µg/ml collagenase type I (Sigma-Aldrich, Poole, UK). After centrifugation, cellular pellets were washed 3 times in fibroblast medium RPMI-1640, 10% FCS, 2 mM glutamine and 1% antibiotic-antimycotic (GIBCO, Paisley, UK) and recovered by centrifugation at 833g for 5 min. A further centrifugation at 330g for 5 min was used to separate stromal cell population (supernatant) from epithelial cells (pellet).

Epithelial cells were cultured in keratinocyte serum-free medium (KSM) supplemented with 2 mM glutamine, 50 µg/ml bovine pituitary extract, 5.9 ng/ml epidermal growth factor and 1% antibiotic-antimycotic solution. Stromal cells were cultured in fibroblast medium (RPMI-1640, 10% FCS, 2 mM glutamine and 1% antibiotic-antimycotic).

Northern and western blot analyses

Northern hybridization was performed using human clusterin, histone H3 and Gas1 full-length cDNAs as probes, as previously described.20 Western blot analyses were performed by chemiluminescence, as previously described.10 Clusterin immunoreactive bands were detected with the BM Chemiluminescence Blotting Substrate (POD) (Roche Diagnostics Corporation, Indianapolis, IN) using monoclonal anti-human clusterin antibodies (UPSTATE Biotechnology, Lake Placid, NY).

In situ hybridization

In situ hybridization experiments were performed in parallel on contiguous (serial) cryosections using a 45mer 35S-labeled oligodeoxyribonucleotide probe specific for human clusterin mRNA (5′ CCA TGG CCT GCT GAG CCT GTG TCA TCT CAA GGA AGG GCT GTA 3′). The probe was labeled and used according to the experimental protocol previously described.9 For human histone H3 mRNA localization, 2 different 45mer (5′ TTT ACC ACC GTG CTA TGG GGC GCT CTT CTG TCG AAC AGC CAT 3′ and 5′ ATT TCA CGG AGC AGC ACA GTC CCA GGC CTG CTA CGA TAA CGA GTA TGC TTC 3′) were used in independent experiments, and the results obtained were identical. Also for the localization of human Gas1 mRNA 2 different oligonucleotides, a 45mer (5′ CCG AGG CCG TGG AAA AGT TTG TCC AAG TCC TGC CCA CTT CTT GCA 3′) and a 46mer (5′ ATT ACC ACC GTG CTA TGG GGC GCT CTT CTG TCG AAC AGC CAT 3′), were independently used and gave the same results. For localization of human ODC mRNA, a 45mer oligonucleotide was used (5′ GTC TGC TCA CTC GAC TCA TCT TCG TCA GAG CCC GTC TGT TCC 3′). Clusterin mRNA localization was also performed with an antisense RNA probe. Contiguous sections were hybridized with an 35S-labeled antisense RNA probe obtained by cloning the fragment between positions 146 and 516 of human clusterin mRNA (GenBank accession number X14723) in the vector pGEM-Tz. In vitro transcription driven by the SP6 promoter gave a radioactive antisense RNA probe, while driven by a T7 promoter it gave a radioactive sense RNA that was used as control in contiguous sections. Experimental conditions for in situ hybridization using the antisense RNA probes were as described for the sense sections were routinely examined in parallel with both sense and antisense oligodeoxyribonucleotide or RNA probes. To exclude nonspecific hybridization other than human clusterin mRNA, the in situ experiments were performed including the use of sense controls, different antisense oligonucleotides targeting different regions of the same mRNA and oligonucleotides of the same length and GC content but containing unrelated sequences.9 For clusterin localization, the results obtained by in situ hybridization were compared to those obtained by immunohistochemistry experiments performed in contiguous sections. Digital black-and-white images of the autoradiographs of whole-specimen cross sections were acquired directly by means of a CCD camera after exposure to Hyperfilm-3H (Amersham Pharmacia Biotech, Little Chalfont, UK), while digital high-magnification photomicrographs showing the cellular distribution of human clusterin mRNA, were acquired by means of a CCD camera attached to the microscope, after treatment of the above sections with photographic emulsion (Ilford K5). Counterstaining was performed with hematoxylin, and cover slips were mounted with Eukitt (O. Kindler GmbH & Co., Freiburg, Germany).

Immunohistochemistry

The same monoclonal anti-human clusterin antibody, clone 41D (UPSTATE Biotechnology, Lake Placid, NY), exhibiting specific binding in Western blot to the 53 kDa precursor of human clusterin (see Fig. 1b), was used for immunohistochemistry staining. The entire experimental procedure was performed on contiguous (serial) prostate cryosections as previously detailed.8 All the sections obtained from benign and cancer tissue were processed in parallel under identical reaction conditions. Experiments performed using polyclonal anti-human clusterin antibodies from the same commercial source gave the same pattern of specific distribution. Negative controls, made by excluding mono- or polyclonal anti-human antibodies from the reaction, showed no specific staining. Counterstaining was performed with hematoxylin, and cover slips were mounted with Eukitt (O. Kindler GmbH & Co., Freiburg, Germany).

RESULTS

In a preliminary study conducted by means of Northern and Western blot analyses, the levels of clusterin mRNA and protein (Fig. 1) were assessed in the cancerous portion and matched benign counterparts of prostate specimens from radical prostatectomy of 25 patients that had undergone maximal androgen blockade for 3–6 months before surgery. The results confirmed our previous data that the clusterin gene is generally downregulated in CaP, independently of the degree of malignancy. Under these experimental conditions it must be noticed that, as shown in Fig. 1b, the antibody used (monoclonal anti-human clusterin antibody from UPSTATE Biotechnology, Lake Placid, NY) recognizes the 53 kDa precursor, from which all clusterin isoforms are supposed to derive. Fig. 1a also shows the specific pattern of expression of Gas1 (specific marker of cell quiescence) and histone H3 (specific marker of cell proliferation) genes in well- and poorly differentiated CaPs. In well-differentiated CaPs, the finding of higher Gas1 and lower H3 mRNA than control is consistent with low prolifera-
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In a representative experiment of 9 (performed on 9 different specimens) are shown. For poorly differentiated CaP, a representative experiment of 5 (performed on 5 matched adenocarcinoma counterparts from the same gland. For well-poorly differentiated human CaP specimens. N, benign tissue; C, clusterin signal was high, but its localization was not homogenous. In fact, clusterin signal appeared to decorate only the basal lamina and the periglandular stroma. A typical situation is illustrated in Figure 4a, where it is evident that the protein is distributed only in well-defined areas lining the perimeter of several but not all glands.

In poorly differentiated CaPs, clusterin immunostaining was confined to what appears to be the remnants of the stromal matrix left behind by tumor invasion (Fig. 4f and g). However, small clusters of epithelial cells inside cancer nests exhibited clusterin expression (Fig. 4i). In these transformed cells the expression of clusterin is clearly confined to the cytoplasm. Figure 4e shows a field where the tissue structure has been nearly totally disrupted by tumor cell invasion, and only sparse remnants of stromal, clusterin-positive material have remained, almost lost among the transformed epithelial cell population that is largely negative to clusterin staining.

Semi-quantitative analysis of clusterin localization and level of expression in stromal and epithelial tissue from matched benign and CaP specimens is summarized in Table I.

As shown in Figure 5, clusterin mRNA and protein were both highly expressed by stromal cells in both well and poorly differentiated CaP specimens when grown as primary cultures. In this case, both 70 kDa clusterin precursor and about 45 kDa mature isoforms are expressed. In epithelial cells, obtained from the same tissue samples, clusterin mRNA was almost undetectable, while very little protein was present, mostly in the well-differentiated specimen. The absence of stromal cell contamination in the epithelial cell cultures is demonstrated by the detection of cytokeratins, specifically expressed by epithelial cells, while the absence of epithelial cell contamination in the stromal cell cultures is dem-

**Figure 1** – Representative Northern hybridization (a) and Western blot (b) analyses of clusterin expression in well-differentiated and poorly differentiated human CaP specimens. N, benign tissue; C, matched adenocarcinoma counterpart from the same gland. For well-differentiated CaP, a representative experiment of 5 (performed on 5 different specimens), and for poorly differentiated CaP, a representative experiment of 9 (performed on 9 different specimens) are shown. In a, ethidium bromide staining of total RNA is shown for comparison (10 μg of total RNA was loaded on each lane). In b, the detection of clusterin protein in 4-day castrated rat ventral prostate (RVP 4dC) is also presented for comparison (30 μg of protein was loaded on each lane). Note that monoclonal anti-human clusterin antibody exhibits cross-reaction between rat and human in Western blot analysis. Only the 53 kDa precursor form of clusterin was detected in CaP specimens. The reverse is true in poorly differentiated CaPs, whose proliferative activity is higher than control.

The regional and cellular distribution of clusterin mRNA was studied in sections of human CaP specimens by means of in situ hybridization histochemistry (Figs. 2 and 3). For comparison, the localization of histone H3 and Gas1 mRNA was also assessed in parallel in contiguous sections.

The autoradiograms of whole sections (Fig. 2) indicated that in well-differentiated CaPs, the signal for clusterin mRNA was stronger than in high-grade cancer, and its distribution matched that of Gas1, both transcripts exhibiting stromal localization. The presence of clusterin signal within the stroma of well-differentiated CaPs (and its absence in the epithelium of most glands) is confirmed by a high-power microphotograph (Fig. 3a). For comparison, we also localized, in contiguous sections, the transcript coding for the rate-limiting enzyme of polyamine biosynthesis, ornithine decarboxylase (ODC). As shown in Figure 3b, ODC mRNA is expressed in the epithelium of most glands and not in the stromal compartment. In limited areas of poorly differentiated specimens (Fig. 2), clusterin mRNA appeared to localize also to regions where the histone H3 mRNA was actively expressed. Similarly to well-differentiated CaP, also in poorly differentiated CaP specimens Gas1 mRNA was only present in the stroma, which is reduced as a consequence of tumor cell invasion. Thus, in situ hybridization experiments indicated that in advanced cancer also, clusterin transcript is mainly expressed within the stromal compartment; although in experiments not shown here, we found rare nests of clusterin mRNA-positive epithelial cells.

Immunohistochemistry analysis (Fig. 4) was performed in CaP specimens in comparison to matched benign counterparts using the same antibody previously used for Western analysis. In benign tissue (Fig. 4b) and in well-differentiated CaPs (Fig. 4c), clusterin protein, similarly to its mRNA, localized prevalently to the stroma, while the signal was down to background levels in normal and transformed epithelial cells. Very high levels of clusterin were found in the basal lamina of a few glands from well-differentiated CaPs (Fig. 4c).

In the stromal compartment of moderately differentiated CaP, clusterin signal was high, but its localization was not homogenous. In fact, clusterin signal appeared to decorate only the basal lamina and the periglandular stroma. A typical situation is illustrated in Figure 4e, where it is evident that the protein is distributed only in well-defined areas lining the perimeter of several but not all glands.

In poorly differentiated CaPs, clusterin immunostaining was confined to what appears to be the remnants of the stromal matrix left behind by tumor invasion (Fig. 4f and g). However, small clusters of epithelial cells inside cancer nests exhibited clusterin expression (Fig. 4i). In these transformed cells the expression of clusterin is clearly confined to the cytoplasm. Figure 4e shows a field where the tissue structure has been nearly totally disrupted by tumor cell invasion, and only sparse remnants of stromal, clusterin-positive material have remained, almost lost among the transformed epithelial cell population that is largely negative to clusterin staining.

Semi-quantitative analysis of clusterin localization and level of expression in stromal and epithelial tissue from matched benign and CaP specimens is summarized in Table I.
The stromal distribution of clusterin that we have reported that also in the human prostate clusterin localizes to the prostate fibroblasts, while being almost undetectable in the epithelial cells. Clusterin protein and mRNA are expressed at high levels mainly by cells obtained from the same tissue samples confirmed that indeed in CaP specimens and in primary cultures of epithelial and stromal cells obtained by converging techniques that enabled us to study the ablation was unexpected. Nevertheless, our experimental data, found in prostate specimens from patients undergoing androgen ablation, demonstrated by the detection of vimentin, specifically expressed by fibroblasts.

Figure 6a shows a section from a poorly differentiated CaP specimen with a peripheral nerve whose branches expressed clusterin mRNA at markedly high levels. For comparison, the localization of ODC mRNA is also shown in a contiguous section from the same specimen. Tumor tissue expresses high levels of ODC mRNA (Fig. 6b, right), while the signal was at the background level in the nerve portion (Fig. 6b, center). The region of the peripheral nerve still not invaded by tumor (not expressing ODC mRNA) is characterized by very high levels of clusterin mRNA (Fig. 6a). Thus, clusterin and ODC mRNA signals appear to be distributed in reciprocal fashion in the 2 serial cryosections shown. The reciprocal response of ODC and clusterin genes, with ODC being repressed and clusterin being overexpressed in regressing tissues and cells brought to quiescence, has been previously found in a number of biologic systems.

**DISCUSSION**

In view of the fact that in the castrated rat prostate clusterin is expressed in the epithelial cells of the ducts, and because of reports that also in the human prostate clusterin localizes to the epithelium, the stromal distribution of clusterin that we have found in prostate specimens from patients undergoing androgen ablation was unexpected. Nevertheless, our experimental data, obtained by converging techniques that enabled us to study the levels of both clusterin mRNA and protein (and their localization) in CaP specimens and in primary cultures of epithelial and stromal cells obtained from the same tissue samples confirmed that indeed clusterin protein and mRNA are expressed at high levels mainly by prostate fibroblasts, while being almost undetectable in the epithelial cells. Besides being associated with apoptosis and cell atrophy, clusterin overexpression also occurred in human fibroblasts when brought to quiescence by serum starvation. In the different stages of tumor progression, clusterin accumulated in specific, well-defined areas of the stromal compartment. In low-grade tumors, the accumulation of clusterin in the basal lamina (Fig. 4c) might indicate that the degradation of this structure, a prerequisite for tumor invasion, was ongoing, and that the resulting debris is being removed (see below). The same pattern of clusterin expression was also detected in the basal lamina and in the stroma of small vessels surrounded by neoplastic cells (not shown), suggesting that clusterin staining of these structures could be associated with microdissemination of neoplastic cells. The periglandular distribution of clusterin that we found in several glands of moderately differentiated tumors (Fig. 4e) could be an indication of the molecular damage exerted on the basal lamina and the surrounding stroma by invasion of transformed epithelial cells. In high-grade tumors, clusterin appears heterogeneously distributed in what seems to be the remnants of the stromal compartment resulting from cancer cell invasion, which would cause stroma derangement, quiescence and/or apoptosis of fibroblasts and concomitant clusterin overexpression. Decreased cell proliferation in the stroma of the benign tissue flanked by poorly differentiated CaPs is suggested by Gas1 expression, which is higher when compared to the level of the benign tissue flanked by well-differentiated tumor (Fig. 1a). Consistent with the hypothesis of reduction of stromal tissue by cancer invasion is also the fact that the stroma surrounded by poorly differentiated CaPs is a minor part of the specimen, when highlighted by Gas1 mRNA expression by *in situ* hybridization (Fig. 2). Thus the protein, once secreted in the extracellular matrix, would participate in the stromal remodeling/degradation processes by interacting with other stromal component(s). It could thus play...
related to the spread of cancer cells via the nerve, which may induce a state of stress of the nerve cells leading to clusterin overexpression. Clusterin gene downregulation, observed in well-differentiated as well as poorly differentiated tumors, represents an early event during CaP progression that may result, among other molecular mechanisms, from a specific genetic loss. The clusterin gene is present as a single copy and maps to human chromosome 8p21 in the 8p21 region. It has been hypothesized that one (or more) tumor suppressor genes are located on human chromosome 8, which contains one of the most frequently deleted loci observed in prostate cancer cells, namely, 8p22-8p21. Furthermore, when Dunning rat prostate carcinoma cells were used as recipients for human chromosomes, metastasis suppressor activities were identified on chromosome 8. We have been able recently to show that transient clusterin overexpression in simian virus 40 (SV40)-immortalized human prostate epithelial cells (PNT2 cells), besides inhibiting ODC activity, resulted in the slowdown of cell cycle progression and decrease of DNA synthesis, thus opposing the effect of SV40. Therefore, in such an experimental model clusterin appeared to act as an antioncogene. Consistent with the above data is our present report that the majority of malignant cells from high-grade tumors did not express clusterin (Fig. 4f to g).

Clusterin mRNA and protein was recently shown to be downregulated also in esophageal squamous cell carcinoma, suggesting that this event could be a general response during tumor onset and progression, not only limited to CaP. Currently, the same experimental techniques presented here are being applied in our laboratory on CaP specimens obtained from patients not subjected to androgen ablation therapy before surgery. Preliminary data show that the pattern of distribution of clusterin mRNA and protein in the tumor does not seem to be affected by the treatment. At variance, we found a consistent increase in clusterin signal in the benign counterpart from gland specimens obtained from androgen-ablated patients, compared to benign specimens obtained from nontreated patients.

Our results on clusterin distribution in the stromal compartment and its downregulation in the tumor tissue apparently disagree with data reported by other authors that clusterin expression, induced by androgen ablation in human CaP specimens, exclusively localizes to the cytoplasm of epithelial cells and that clusterin levels are enhanced in tumors. Differences in experimental conditions may have contributed to the production of these seemingly conflicting results. In a part of the use, for immunohistochemistry experiments, of paraffin blocks vs. fresh tissue immediately frozen, used in the present study, what really can make a difference is the utilization of antibodies from different commercial sources. Our results were obtained with a monoclonal antibody (UPSTATE Biotechnology, Lake Placid, NY), while the antibody used by July et al. is a polyclonal one (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Thus, an explanation of the discrepancy between our results on the clusterin localization and those of July et al. may be found in the hypothesis that different forms of clusterin are present in the stromal and epithelial compartments and that the antibody used by us recognizes the stromal form but not the epithelial one, while the antibody from Santa Cruz only recognizes the epithelial form. By the same means, the fact that we show the levels of clusterin to be lower in cancer than in the benign counterparts, but others show it to be higher, may be understood if we hypothesize that the stromal form of clusterin decreases because of stromal destruction by cancer progression, while the putative epithelial form, present in the transformed cells, increases with tumor progression.

Since our data on in situ hybridization and immunohistochemistry experiments show that clusterin mRNA localizes to the stromal compartment similarly to clusterin protein, while evidence for the presence of clusterin transcript within the epithelium is lacking, one can speculate that clusterin is originally produced by the stromal cells and, after being released to the extracellular matrix,
is taken up by the epithelial cells and rapidly processed to produce the (antiapoptotic?) epithelial form of the protein. This would represent an example of cross-talk between stroma and epithelium.

Several forms of clusterin have been demonstrated to derive from a single mRNA. This may explain why this glycoprotein appears to play totally different functions and participate in processes that may have opposite effects on the cell. An example of this ambiguity is clusterin involvement in the processes of cell death and cell survival. The rare and scattered nests of transformed epithelial cells expressing clusterin that have been found in high-grade cancers (Fig. 4, arrow) are undistinguishable from the majority of transformed epithelial cells that do not express the glycoprotein. A tentative interpretation of this finding could be that these cells exhibit a constitutive, intracellular expression of the form of the protein that is involved in cell survival and inhibition of apoptosis. Thus, these cells could represent those responsible for tumor relapse.

Since CaP is a heterogeneous polyclonal disease, in which normal and transformed cells respond differently to hormonal environment and therapy, we suggest that tumor growth, under conditions of long-term androgen blockade, could lead to down-

TABLE 1 – EXPRESSION OF CLUSTERIN IN PROSTATE TISSUES

| Case group                     | Total | Gleason score | Stroma (positive) | Staining intensity¹ | Epithelium (positive) | Staining intensity¹ |
|-------------------------------|-------|---------------|-------------------|---------------------|-----------------------|---------------------|
| Benign                         | 25    | –             | 20                | +                   | 6                     | ±                   |
| Cancer, well differentiated    | 5     | 2–4           | 5                 | +                   | 0                     | –                   |
| Cancer, moderately differentiated | 11    | 5–6           | 9                 | ++/+++              | 0                     | –                   |
| Cancer, poorly differentiated  | 9     | 7–10          | 8                 | +++                 | 0                     | –                   |

¹Staining intensity: +++, strong; ++, moderate; +, weak; ± only occasional staining; –, no staining.

FIGURE 4 – Immunohistochemistry detection of clusterin protein in cryosections from human CaP specimens. (a and b) Benign tissue; (c) well-differentiated CaP; (d and e) moderately differentiated CaP; (f–i) poorly differentiated CaP. (a) Negative control (10×); (d) clusterin immunostaining in benign tissue (section consecutive to that of a, 10×); (c) clusterin immunostaining in stroma and basal lamina of well-differentiated CaP at high magnification (20×); (d) negative control of moderately differentiated CaP at high magnification (20×); (e) clusterin immunostaining of moderately differentiated CaP at high magnification (20×), in which it is shown that epithelial cells are negative, while clusterin staining is clearly periglandular (but nonhomogeneous) in the whole section. In fact, the stroma at close contact with some glands is clearly positive for clusterin (center), while the stroma surrounding other glands, exhibiting morphologic features apparently identical to the previous ones, is negative (left and right). (f) Clusterin immunostaining is confined to stromal remnants in poorly differentiated CaP (20×). (g) Same section, different field of f (40×), showing that the remnants of stromal extracellular matrix, subjected to invasion by tumor cells, are positive for clusterin immunostaining, while the vast majority of transformed epithelial cells are negative. (h) Stromal and basal membrane invasion and disruption, which is almost completed in this specimen of invasive and poorly differentiated adenocarcinoma, are associated with clusterin expression in stromal remnants and clusterin downregulation in the majority of neoplastic epithelial cells (20×). (i) Same section, different field of g (40×), in which a nest of transformed epithelial cells, positive for clusterin staining, is indicated (arrow). The data are representative of a trend in clusterin expression shown by a panel of 25 patients. Counterstaining was performed with hematoxylin.
regulation of clusterin gene expression in the CaP tissue; (ii) localized, stromal periglandular increased expression, in low-grade tumors, of the extracellular isoform of clusterin that is actively involved in the process of stromal remodeling caused by tumor invasion; and (iii) possible selection of a minor population of transformed epithelial cells in which the clusterin gene becomes relaxed, resulting in the constitutive overexpression of the anti-apoptotic form of this protein, which would confer surviving features to these cells.

The development and purification of a panel of antibodies capable of recognizing structurally distinct isoforms of human clusterin is currently in progress in our laboratory. The completion of this project will enable us to study the intra- and extracellular processing of clusterin protein family members, testing the hypothesis that different forms of clusterin, all derived from the same gene, could play distinct functions in normal and pathologic prostates. Our study could disclose the potential advantages of using the detection of specific human clusterin isoforms as molecular markers of CaP progression for the clinical management of this disease.

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