

STEREOLOGIC ESTIMATION OF KI-67, CASPASE 3 AND GSTP1 POSITIVE CELLS IN PROSTATE LESIONS

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ABSTRACT

Cell proliferation, caspase 3 and pi-form of glutathione S transferase (GSTP1) were evaluated in prostate carcinoma (PCA), proliferative inflammatory atrophy (PIA) and prostate intraepithelial neoplasia (PIN). Forty biopsies were classified as: without morphological lesions (controls: CTR), PIA, PIN and PCA. Ki67, caspase3 and GSTP1 were immunostained. The following estimates were performed: Numerical densities of Ki67+ cells ($N_V\text{EPKi67}$), of all epithelial cells ($N_V\text{EPtotal}$) and of GSTP1+ cells ($N_V\text{EPGSTP1}$); labelling index for Ki67 (LI_{Ki67}); volume fraction to caspase 3 positive tissue ($V_V\text{caspase 3}$) and of GSTP1 positive tissue ($V_V\text{GSTP1}$). ANOVA was performed to compare the groups. $N_V\text{EPtotal}$ and $N_V\text{EPKi67}$ were increased in PIA. LI_{Ki67} was only increased in PCA. $V_V\text{caspase 3}$ was decreased in PIN and PCA. $V_V\text{EGSTP1}$ was decreased in PCA. In our results PIA lacks the characteristics of a premalignant lesion. The result may be explained by the use of unbiased quantitative methods, the inadequate definition of PIA and the scarce inflammation observed in the samples with PIA included in this study.

Keywords: caspase 3, cell proliferation, GSTP1, Ki-67, preneoplasia, prostate, stereology.

INTRODUCTION

Prostate carcinoma (PCA) is the most common type of cancer among elderly men and is the second most frequent cause of cancer death in the United States (Tsujimoto *et al.*, 2002). From the perspective of protection, early prevention and treatment for PCA, recognition of precursor lesions is important. Prostate intraepithelial neoplasia (PIN), particularly high-grade PIN, has been reported to be the most likely precursor lesion for PCA (McNeal *et al.*, 1986; Myers *et al.*, 1994; Bostwick *et al.*, 1995; Emmert-Buck *et al.*, 1995).

Proliferative inflammatory atrophy (PIA) of prostate acini consists of clusters of small acini that are atrophic in appearance and surrounded by stromal reaction with chronic inflammatory infiltrate. PIA changes have been reported to show increased proliferative activity compared with simple atrophy (Feneley *et al.*, 1996; Ruska *et al.*, 1998). Recently, Putzi and De Marzo (2000) reported the morphologic transition between high-grade PIN and PIA, and suggested that proliferative epithelium in PIA might progress to PIN and/or PCA.

A study on tissue homeostasis has confirmed that the kinetics of tissue growth is contingent on two independent parameters: the rate of cell proliferation and the rate of cell death (apoptosis) (Colombel *et al.*, 1998). The balance between cell proliferation and apoptosis can be altered in prostate cancer, and it is known that programmed cell death occurs under genetic control and that defects in apoptosis may be associated with the development of cancers (Harriss *et al.*, 1995).

Caspases are fundamental components of the mammalian apoptotic machinery. Caspase 3 is a prototypical enzyme that becomes activated during apoptosis in a wide variety of tissues (Woo *et al.*, 1998). On the other hand, there is evidence of loss of caspase 3 protein expression in prostate cancer (Winter *et al.*, 2001). The quantification of immunoexpression of caspase 3 may constitute a good method for measuring apoptotic activity in prostate cancer and its precursors.

The glutathione S-transferases (GST) are an important class of enzymes that play a prominent role in the intracellular detoxification of products of oxidative stress by catalyzing the conjugation of these

compounds to glutathione (Parsons *et al.*, 2001). The most extensively studied of these GSTs in the human prostate is the pi-form of GST (GSTP1). Most basal cells in normal prostate epithelium express GSTP1 (Lee *et al.*, 1994; Cookson *et al.*, 1997); the great majority of prostate cancer cells, however, fail to express GSTP1 due to hypermethylation of the GSTP1 gene promoter (Nakayama *et al.*, 2003; Nakayama *et al.*, 2004), and 70% of high-grade PIN lesions (Parsons *et al.*, 2001) show decreased expression of GSTP1, suggesting that the decreased expression of this enzyme renders prostate cells vulnerable to malignant progression. On the other hand, prostate lesions consisting of both simple and inflammatory proliferative atrophy express elevated levels of GSTP1 protein in secretory luminal cells (De Marzo *et al.*, 1999). It is possible that increased expression of GSTP1 in PIA may result from the presence of an ongoing oxidative insult to this tissue, while silencing of GSTP1 function might be related to development of cancer (Parsons *et al.*, 2001). An adequate quantification of proliferative, apoptotic and GSTP1 activities seems relevant in order to establish the significance of the presumptive premalignant character of both PIA and PIN lesions.

The proliferative status of malignant or premalignant lesions has been studied using semiquantitative methods for estimation of BrdU incorporation, PCNA and Ki67 labelling indices, AgNOR quantification, etc., and mitotic counts (Limas *et al.*, 1994; Hofstadter *et al.*, 1995; Bubendorf *et al.*, 1996; Bubendorf *et al.*, 1998). Apoptotic activity has been frequently evaluated by counting apoptotic bodies, or detecting in situ end-labelled fragmented DNA (Brown *et al.*, 1996; Deng *et al.*, 1996). The degree of immunohistochemical staining for GST was evaluated for some authors using a stereological point-counting method that was not very well specified (Parsons *et al.*, 2001).

Summarizing, the study of references (Limas *et al.*, 1994; Hofstadter *et al.*, 1995; Brown *et al.*, 1996; Bubendorf *et al.*, 1996; Deng *et al.*, 1996; Bubendorf *et al.*, 1998; Parsons *et al.*, 2001) regarding evaluation of the aforesaid markers does not show that evaluation was carried out in an unbiased manner or reliably from the stereological point of view. In the present study, we undertake a rigorous quantitative stereological estimate of the numerical density (N_V) of proliferating cells and labelling index for Ki67, volume density (V_V) of caspase 3 epithelial immuno-reactivity, and N_V and V_V for GSTP1 immunostained epithelial cells, in prostate needle biopsies without lesions, with PIA, with high-grade PIN and with PCA. Our goal is to determine whether the stereological evaluation of

prostate pathology can confirm the findings to date on these markers.

MATERIAL AND METHODS

Forty biopsies performed by transrectal ultrasound guided prostate needle biopsy were collected from 40 patients screened for PCA because they presented high blood levels of prostate-specific antigen (PSA). The patients were studied at the N. Sra Sonsoles Hospital, Ávila, Spain, during the period from 2000 to 2001. The age of the patients ranged from 56 to 85 years (mean 70 yr). The biopsies were classified into 4 groups (10 per group): samples without any morphological lesion (CTR), samples with inflammatory atrophy (PIA), samples with high-grade PIN (PIN) and samples with prostate adenocarcinoma (PCA). The PCA cases were without prior neoadjuvant hormonal therapy. The biopsies included in each group had only the pathology ascribed to that group; e.g., the specimens from the PIN group had exclusively PIN lesions, and no other pathology.

The specimens were fixed in 10% paraformaldehyde in PBS, pH 7.4, for 24 hrs, and processed for paraffin embedding. The paraffin blocks were exhaustively sectioned at 15 μ m, although thicker sections could be desirable for the optical disector (Howard and Reed, 1998). However, the small size of the biopsies meant that thicker sections could not be taken from biopsies, which were also used for diagnostic purposes. A total of 20 sections per specimen were systematically randomly sampled (Gundersen *et al.*, 1988) from all sections obtained in each biopsy. A fraction of these sections, again systematically randomly sampled, were immunostained for Ki67, caspase 3 and GSTP1, and a total of five sections per each antibody were finally studied.

Histological sections were reviewed independently by two pathologists (R.M. and L.S.). The diagnosis of PIA was based on earlier criteria (De Marzo *et al.*, 1999) as follows: a microscopic cluster of small acini with irregular atrophic-appearing contours lined by cuboidal cells, surrounded by stroma with chronic inflammatory changes. Only high-grade PIN was considered, based on cellular atypia of intraluminally proliferated cells (Bostwick *et al.*, 1987). PCA was included as samples of different Gleason grades (Veterans Administration Cooperative Urological Research Group and Gleason DF, 1977).

Deparaffinized and rehydrated tissue sections were treated for 30 min with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS), pH 7.4, to block

endogenous peroxidase. Mouse monoclonal and rabbit polyclonal antibodies were used as primary antibodies. To detect Ki67 and caspase 3 immunoreactivities, sections were incubated with a monoclonal anti-Ki67 antibody (Ventana Medical Systems, Tucson, AZ, USA) diluted 1:1,000 and with a monoclonal anti-caspase 3 antibody (Cell Signaling Technology Inc., Beverly, MA, USA) diluted 1:50, respectively. To detect GSTP1 immunoreactivity, sections were incubated with a polyclonal anti-GSTP1 antibody (Novocastra, Newcastle, UK) diluted 1:100. Pre-treatment of sections by heat in a citrate buffer (using a pressure cooker; Martin *et al.*, 2001) was performed to enhance all immunostainings. All primary antisera were diluted in PBS pH 7.4 containing 1% bovine serum albumin (BSA) plus 0.1% sodium azide. All incubations with primary antisera ran overnight at 4° C. When primary monoclonal antibodies were used, the second antibody employed was a biotin- caproyl-anti-mouse immunoglobulin (Biomed, Foster City, CA, USA). In addition to the primary polyclonal antibody, the second antibody used was a biotin-caproyl-anti-rabbit immunoglobulin (Biomed). Secondary antibodies were diluted 1:400 in PBS containing 1% BSA without sodium azide and incubated for 30 min at room temperature. Thereafter, sections were incubated with a streptavidin-biotin-peroxidase complex (Biomed). The immunostaining reaction product was developed using 0.1 g diaminobenzidine (DAB; 3,3',4,4'-Tetraminobiphenyl, Sigma, St Louis, MO, USA) in 200 ml of PBS, plus 40 µl hydrogen peroxide. After immunoreactions, sections were counter-stained with Harris haematoxylin. The sections selected for measuring caspase 3 and GSTP1 immunoreactivities were not counterstained. All slides were dehydrated in ethanol and mounted in Depex (Serva, Heidelberg, Germany). Specificity of the immunohistochemical procedures was checked by incubating some sections not sampled for measurements with nonimmune serum instead of the primary antibody.

The following features were evaluated from the sections:

- (i) Number of epithelial cells with nuclei immunoreactive to Ki67 per unit of epithelial volume ($N_{V\text{EPKi67}}$).
- (ii) Number of total epithelial cells (immunostained and not immunostained for Ki67) per unit of epithelial volume ($N_{V\text{EPtotal}}$).
- (iii) Number of epithelial cells immunoreactive for GSTP1 per unit of epithelial volume ($N_{V\text{EPGSTP1}}$).

Estimation of the N_V was performed using the technique of optical disector, which is an unbiased stereological method (Wreford, 1995; Howard and Reed, 1998). Five 15-µm-thick sections per immunostaining (Ki67, GSTP1) were chosen by systematic random sampling from each 20 sections sampled from the pool of sections obtained by complete sectioning of each specimen. Measurements were carried out using an Olympus microscope equipped with a $\times 100$ oil immersion lens (numerical aperture of 1.4) at a final magnification of $\times 1,200$ and using the stereologic software CAST-GRID (Interacti-vision, Silkeborg, Denmark; Rodriguez *et al.*, 2003). This program allows the selection of fields to be studied by random systematic sampling after the input of an appropriate sampling fraction. An average of 20 fields/section was selected and used to count the number of cells; the counting unit was the nucleus, assuming the mononucleated character of every cell. An average of five cell (nuclei) profiles (range 1–13) per microscopic field (disector frame of $1,503 \mu\text{m}^2$) was collected for stereological estimations. The entire area of the sections was included in the sample fields in each group. Only the areas with a lesion were scanned. The numerical densities were expressed as follows: $N_{V\text{EPKi67}}$ = Number of epithelial cells (nuclei) immunoreactive to Ki67/epithelial acinar volume in mm^3 ; $N_{V\text{EPtotal}}$ = Number of epithelial cells (nuclei) positive and negative to Ki67/epithelial acinar volume in mm^3 ; $N_{V\text{EP GSTP1}}$ = Number of nuclei from epithelial cells with cytoplasm immuno-reactive to GSTP1/epithelial acinar volume in mm^3 . The ratio $N_{V\text{EPKi67}}/N_{V\text{EPtotal}}$, expressed as percentage, was the Ki67 labelling index (LI_{Ki67}).

Volume density – *i.e.*, the ratio between the area occupied by a phase or tissue component and a reference area, (V_V) – was employed in the 4 groups studied to estimate the volume fractions of epithelial cytoplasm immunostained either by caspase 3 ($V_{V\text{caspase3}}$) or by GSTP1 ($V_{V\text{GSTP1}}$), over the epithelial acinar volume. The measurement of both immunoreactive and reference areas were estimated as follows: Five sections per each immunostaining were chosen by systematic random sampling from each of the 20 sections sampled from the pool of sections obtained by complete sectioning of each specimen. Measurements were carried out using the same stereologic system employed to estimate numerical densities. This system makes it possible to capture the fields to be measured that were previously selected by random systematic sampling. An average of 20 fields/section were captured using a TK-C1480B colour videocamera (JVC, Japan) with a resolution of

480 TV lines, and images were digitized at 752×582 with 256 grey levels. Grey-level image transformation and binary image processing were carried out using the public domain NIH Image program, developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/> (Martin *et al.*, 2001). An average of 50 cell (nuclei) profiles (range 20–60) per microscopic field ($3,000 \mu\text{m}^2$) were collected for image analysis. The images were manually thresholded, and microscopic analysis was performed by one observer (LS) excluding the possibility of determining inter-observer variability. The stromal compartment was manually discarded from the field studied, and the ratio of epithelial immunostained area was automatically measured by the program in order to obtain either $V_V\text{caspase 3}$ or

$V_V\text{GSTP1}$. The entire area of the sections was included to sample the fields in the CTR group, whereas for the PIA, PIN and PCA groups only the areas with the corresponding lesion were scanned.

For each group, the mean \pm SD was calculated for all the parameters studied. The heterogeneity of the distribution of the markers all over the sections of the lesions studied was assessed obtaining the coefficient of variation (CV) for N_V and V_V estimates between the sections, an average of 25% was obtained for CV in numerical density estimates, with a range from 10% ($N_V\text{EPGSTP1}$ in the PCA group) to 39% ($N_V\text{EPGSTP1}$ in the PIA group), and an average of 26% was obtained for CV in volume fraction estimates, with a range from 19% ($V_V\text{caspase3}$ in PCA lesions) to 36% ($V_V\text{caspase 3}$ in the PIA group).

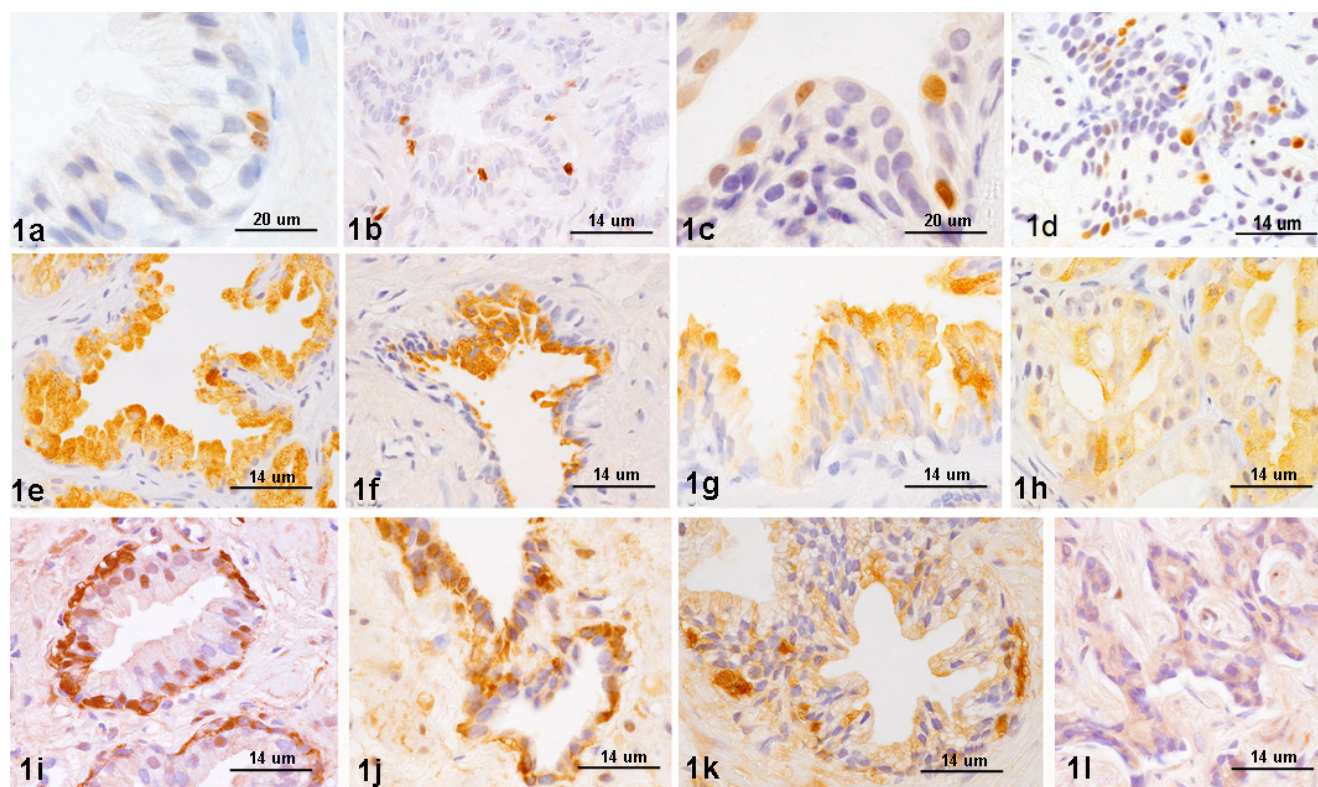


Fig. 1. a) Prostate control immunostained for Ki67; two nuclei immunoreactive from basal cells are seen. b) PIA acini immunostained for Ki67; several immunoreactive nuclei were observed in the atrophic epithelium. c) Several apical nuclei from a PIN lesion show immunoreactivity for Ki67. d) PCA case. The tumour shows some nuclei immunostained to Ki67. e) Specimen from CTR group immunostained to caspase 3. The immunoreactivity was preferentially localized in the cytoplasm of columnar cells. f) PIA gland immunostained for caspase 3. The immunoreactivity was remarkable but irregularly distributed. g) The caspase 3 immunoreactivity in a PIN lesion was scanty and preferentially located at the apical border of the columnar cells. h) PCA specimen. Some isolated cells from cancer infiltrate show caspase 3 immunoreactivity. i) Control prostate glands immunostained to GSTP1. The immunoreactivity was mainly located in the cytoplasm of the basal cells. j) PIA gland immunostained to GSTP1. A remarkable immunoreactivity was observed in the cytoplasm of atrophic epithelial cells. k) PIN gland immunostained to GSTP1. The immunoreactivity was mainly detected in a few basal cells. l) PCA immunostained to GSTP1. A faint immunoreactivity was detected in some cells.

One-way ANOVA was performed in order to ascertain differences among the groups considered for each parameter studied. The Newman Keuls test for multiple comparison of means was performed to search for differences among means, and the level of significance was $p < 0.05$ in all cases.

RESULTS

Occasional immunoreactive nuclei for Ki67 were observed in CTR acini, preferentially located in the basal compartment, (Fig. 1a). A variable amount of nuclei immunostained for Ki67 were detected in PIA

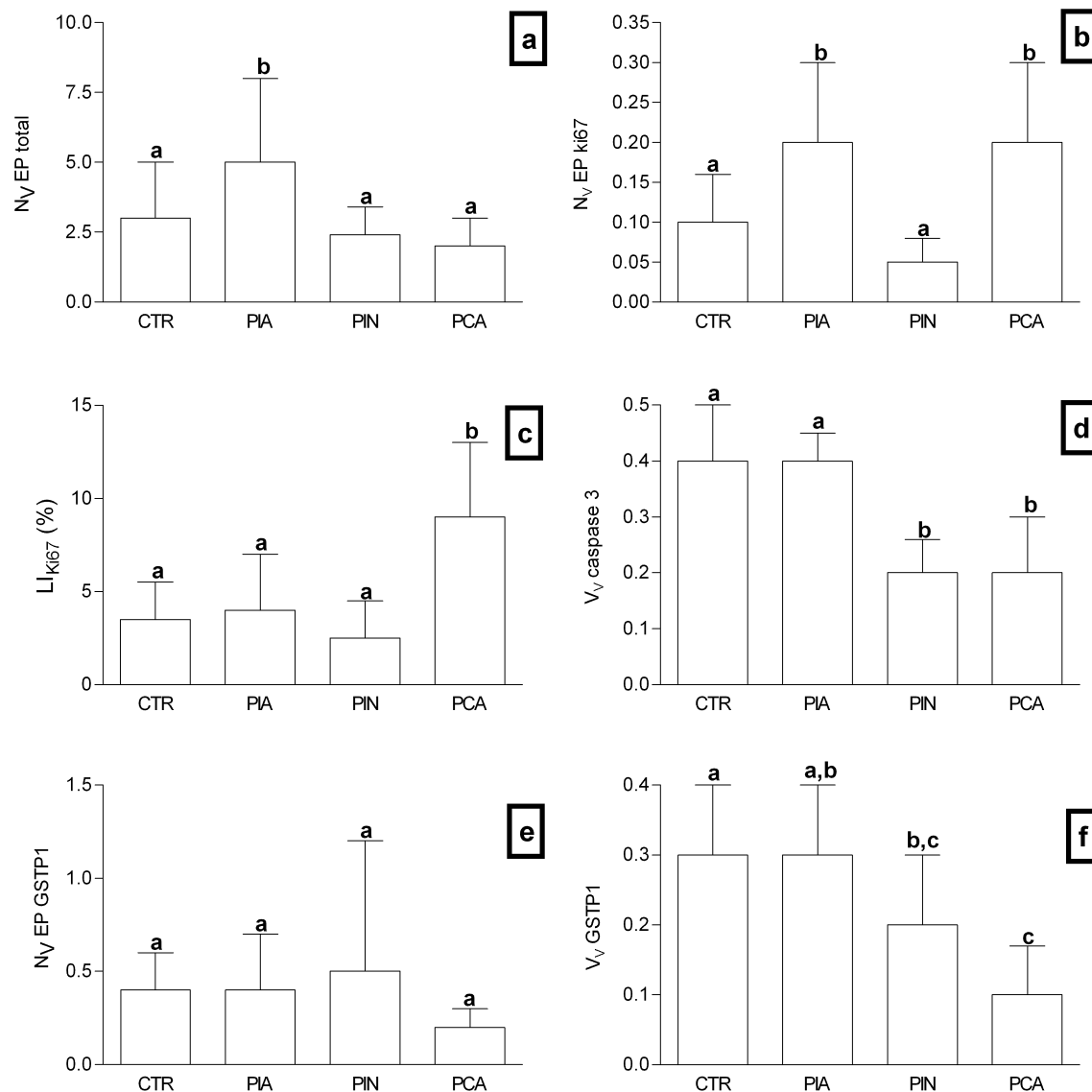


Fig. 2. Bar diagrams indicating the mean \pm SD of the estimated parameters in CTR, PIA, PIN and PCA groups. Bars showing different letters at the top of the error bars differ significantly ($p < 0.05$). a) Numerical density ($N_V EP_{total}$) of epithelial total cells (immunostained and not immunostained for Ki67) expressed as number of cells per mm^3 of epithelial acinar volume. b) Numerical density ($N_V EP_{Ki67}$) of epithelial cells positively immunostained for Ki67 expressed as number of cells per mm^3 of epithelial acinar volume. c) Ki67 labelling index (LI_{Ki67}) of epithelial cells. d) Volume fraction of caspase 3 immunostained cytoplasm ($V_V \text{ caspase 3}$). e) Numerical density ($N_V EP_{GSTP1}$) of GSTP1 immunostained epithelial cells expressed as number of cells per mm^3 of epithelial acinar volume. f) Volume fraction of positively stained cytoplasm from epithelial cells ($V_V \text{ GSTP1}$) immunostained for GSTP1.

lesions, (Fig. 1b), whereas in PIN lesions a higher number of nuclei expressing Ki67 were detected in both basal and columnar cells (Fig. 1c). The amount of nuclei immunostained for Ki67 was prominent in PCA (Fig. 1d).

Immunoreactivity for caspase 3 was abundant in CTR specimens, being more remarkable in the columnar cells (Fig. 1e). PIA glands show also relevant immunoreactivity to caspase 3 (Fig. 1f). However, the PIN group (Fig. 1g) and, more importantly, the PCA group displayed a clear decrease of caspase 3 immunostaining, and immunoreactivity was confined to the apical border of the tumour cells (Fig. 1h). In both the CTR and PIA groups, immunoreactivity to GSTP1 was observed in basal cells of the acini, and there was no difference between the two groups in the intensity of immunostaining (Fig. 1i,j). In the PIN group the GSTP1 immunostaining was decreased, and only scarce basal cells were immunoreactive (Fig. 1k). Cytoplasmic immunoexpression to GSTP1 was scarce and faint in tumour cells from the PCA group (Fig. 1l).

The numerical density of epithelial cells, including proliferating and not proliferating elements ($N_V E_{total}$), was significantly higher in the PIA group than in the other groups. No significant differences were observed between CTR, PIN and PCA cases (Fig. 2a). The numerical density of cells immunostained for Ki67 ($N_V EP_{Ki67}$) was significantly higher in PIA and PCA groups than in CTR and PIN groups. The small difference between CTR and PIN groups was not significant (Fig. 2b). LI_{Ki67} was significantly increased in PCA in comparison with the other groups. Significant differences were not detected between CTR, PIA or PIN groups (Fig. 2c).

Volume fraction of cytoplasm immunostained for caspase 3 ($V_V caspase3$), was significantly decreased in both PIN and PCA cases, in comparison with CTR, and PIA groups. There was no significant difference between CTR and PIA, or between PIN and PCA (Fig. 2d).

There was no significant difference between groups in relation to the numerical density of cells immunoreactive to GSTP1 ($N_V EP_{GSTP1}$; Fig. 2e). However, the volume fraction of cytoplasm immunostained for GSTP1 ($V_V EP_{GSTP1}$) was significantly decreased in the PCA group when compared with either CTR or PIA cases. There were no statistically significant changes between CTR and PIA, or between PIA and PIN, or between PIN and PCA (Fig. 2f).

DISCUSSION

A number of authors (De Marzo *et al.*, 1999; De Marzo *et al.*, 2003; Ruska *et al.*, 1998; van Leenders *et al.*, 2003) have remarked that PIA changes are hyperproliferative and postulate that PIA should be included as a precursor to prostate cancer. However, in the present study, the LI_{Ki67} of PIA lesions estimated as a ratio between the numerical density of Ki67 immunoreactive cells and the numerical density of both proliferative and not proliferative cells does not show significant differences with control acini, although cell density of Ki67 immunoreactive nuclei was higher in the PIA group than in the CTR group. Thus no significant abnormal cell proliferation was found in PIA changes. This might be explained by differences in the methods of measuring, although the techniques of measurement employed in other studies are not clearly described (Ruska *et al.*, 1998; De Marzo *et al.*, 1999; Tsujimoto *et al.*, 2002; De Marzo *et al.*, 2003; van Leenders *et al.*, 2003). The usual methodology for evaluating proliferating indices employs the counting of immunoreactive and non-immunoreactive nuclei profiles per microscopic field (Limas *et al.*, 1994; Hofstadter *et al.*, 1995; Feneley *et al.*, 1996). These methods are not free of bias (Gundersen *et al.*, 1988; Wreford, 1995; Howard and Reed, 1998), whereas the stereological methods performed in this study provide a rigorous quantitative tool, without bias and with correct sampling. On the other hand, proliferative inflammatory atrophy of the prostate is a concept employed in different ways by different authors (Ruska *et al.*, 1998; De Marzo *et al.*, 1999; 2003). Sometimes prostatrophic hyperplasia is included in the definition of PIA (Putzi *et al.*, 2000; Tsujimoto *et al.*, 2002). In the present study, PIA is defined as changes with discrete foci of glandular epithelium with the morphological appearance of simple atrophy (without signs of postatrophy hyperplasia) occurring in association with inflammation. The inflammatory infiltrate observed in almost all the specimens studied was chronic and not very prominent. The scarcity of this inflammatory reaction might also explain the low proliferative indexes. The intensity of chronic inflammation seems to be associated with the carcinogenetic potential of the PIA changes (Zha *et al.*, 2001; Wang *et al.*, 2004).

The changes observed in other parameters considered (apoptotic activity and GSTP1 expression) agree with the idea of the similarity of PIA with control acini. Apoptosis evaluated by quantification of caspase 3 immunoreactivity was importantly decreased in PIN and PCA groups, as indicated by other authors

(Winter *et al.*, 2001), whereas CTR and PIA did not show changes. Again, these findings emphasize the premalignancy of PIN, but did not assign this precursor character to PIA.

GSTP1 (as a marker of oxidative stress resistance) expression has been described higher in PIA than in the normal prostate (Parsons *et al.*, 2001; De Marzo *et al.*, 2003). However, our stereological results show that the numerical density of GSTP1 immunoreactive cells was similar in all the groups studied (CTR, PIA, PIN and PCA), and the amount of immunoreactivity measured as a volume fraction of GSTP1 immunostained cytoplasm was significantly lower in PCA than in both PIA and CTR cases. The position of PIN was intermediate between PCA and PIA. These findings suggest that the loss of response to oxidative stress was more related to the decrease of GSTP1 expression than to the diminution of number of immunoreactive cells. This event is remarkable in prostate carcinoma, as has been stated in other studies (Cookson *et al.*, 1997; Parsons *et al.*, 2001), but our findings in relation to PIA and PIN cases did not agree with these authors (Cookson *et al.*, 1997; Parsons *et al.*, 2001). In contrast with what was observed in other studies (Cookson *et al.*, 1997) GSTP1 was importantly, but not exclusively, expressed in basal cells because a faint but measurable immunostaining was detected in some columnar cells, even in those from cancer acini. This may indicate that hypermethylation of the GSTP1 gene promoter is not complete in all cancer cells (Nakayama *et al.*, 2004).

In conclusion, our stereological quantitative results (cell proliferation, caspase 3 and GSTP1 expression) suggest that PIA glands are more similar to normal glands than to PIN, lacking the characteristics of premalignant lesions. This finding, which is in disagreement with other current opinions, may be explained by (i) the use of unbiased quantitative methods, (ii) the poorly defined concept of PIA lesion and (iii) the scarcity of inflammatory reaction observed in the PIA changes in the present study.

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