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ORIGINAL ARTICLE

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Kinetic profiles of intraepithelial and invasive prostatic neoplasias: the key role of down-regulated apoptosis in tumor progression

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Abstract The cell kinetic of prostatic intraepithelial neoplasia (PIN) is poorly understood. Herein we report the kinetic pattern of PIN, both not associated (primary) and associated (secondary) with coexistent invasive carcinoma (PCa). Surgical specimens collected in 20 cases of primary PIN, 20 of secondary PIN and 20 of PCa were studied by MIB-1 immunostaining, in situ end-labeling (ISEL) and DNA histogram analysis, and the cell density in each case was estimated using the formula $N=(n\pi/4)^2$. Fifty high-power fields (HPF), or the complete lesion if smaller, were screened in each lesion, and both mean and standard deviation were recorded. Statistical differences were studied by means of Fisher's exact test. ISEL indices were significantly ($P<0.0001$) lower in PCa (0.1±0.3) than in primary PIN (0.5±0.3), while the MIB-I indices were similar in both conditions ($P=0.56$). Statistically significant differences were also detected for both MIB-1 and ISEL indices when secondary PIN (MIB-1 1.9±0.7, ISEL 3.7±3.3) was compared with primary PIN (MIB-1 2.5±2.1, ISEL 0.5±0.3) and PCa ($P<0.0001$). In terms of cellularity, primary PIN (26.3±7.1) revealed scores significantly lower ($P<0.0001$) than those recorded in PCa (39.0±8.8) and secondary PIN (32.9±14.3). In conclusion, early prostatic tumor is mainly defined by down-regulated apoptosis rather than by increased proliferation. Secondary PIN displays unique kinetic features suggesting an evolved stage of primary PIN.

Key words Prostatic intraepithelial neoplasm · Precancerous lesion · Intraductal extension · Cell kinetics · DNA-ploidy

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Introduction

Currently, high-grade prostatic intraepithelial neoplasia (PIN) is the most likely precursor of invasive prostatic carcinoma (PCa) [2, 3] and has been reported both as an isolated finding (primary PIN) and coexisting with invasive adenocarcinomas (secondary PIN) [4, 22].

The normal cellular turnover is maintained by a strict balance between proliferation and apoptosis [27, 43], studied by several techniques including immunohistochemistry, in situ end-labeling (ISEL) of fragmented DNA, and DNA-ploidy analysis. Controversial results have been reported on cell kinetics in PIN because of the heterogeneity of cases (especially regarding total androgen ablation) [32, 34, 35] and the diversity of techniques and quantification methods for both proliferating cells [23, 33, 45] and apoptotic cells [18, 31–34, 47]. Additionally, no attempts to differentiate primary from secondary PIN have been published. Therefore, the biological significance of both types of PIN remains unknown, and their potential implications for therapeutic approaches need to be determined.

The purpose of this study was to characterize the kinetic features of both primary and secondary PIN in a series of surgical specimens. Combined quantitative analyses of proliferation and apoptosis markers on tissue sections were performed. Ultimately, those parameters were to help define the kinetic of tumor progression in PCa.

Materials and methods

Case selection

Forty consecutive patients were included in this study, 20 with PIN only (in a background of nodular hyperplasia) and 20 with coexistent low-grade PCa and PIN in the same specimen. All patients showed enlarged prostate and high serologic levels of prostate-specific antigen (PSA); either radical prostatectomy (all patients with PCa and 16 without PCa) or transurethral resection (4 patients without PCa) was undertaken as appropriate treatment. None of these patients underwent androgen deprivation therapy before the surgical resection, and the treatment was selected according to clinicopathological criteria, such as PSA levels or imaging analysis. No histological evidence of extraprostatic tumor extension was detected, and no patient with primary PIN developed pathologically proven PCa during the study (mean follow-up of 92 months).

The surgical specimens were routinely processed and representative samples were taken (following the standard criteria of sampling in prostatic resections) to establish the histological diagnosis [24]. Bostwick's criteria were used to classify high-grade PIN [4]. Appropriate archival material was available for additional studies in each case. The same microscopic areas were evaluated with every technique, and at least five different topographic areas were screened per patient and pathologic condition (primary PIN, PCa, and secondary PIN), 50 HPF or the whole foci being evaluated from each. All these scores were recorded and considered independently to validate the results per patient and per pathologic condition.

MIB-1 immunostaining

The sections were mounted on positively charged microscope slides (Superfrost Plus, Menzel, Germany) and baked at 60°C for 2 h. After routine dewaxing (xylene), rehydration (ethanol) and endogenous peroxidase quenching (0.5% H_2O_2 in methanol, 1–0 min), the sections were microwaved in 10 mM citrate buffer, pH 6.0, for 20 min and serially incubated with polyclonal horse serum (1/100 dilution, Dako Denmark) for 20 min, specific primary antibody (4 µg/ml, Dianova Hamburg, Germany) overnight at 4°C, biotinylated anti-mouse antibody (1/200 dilution, Dako Denmark) for 30 min, and peroxidase-labeled avidin–biotin complex (1/100 dilution, Dako Denmark) for 60 min. All incubations were performed in a moist chamber at room temperature unless otherwise specified. The reaction was developed under microscopic control, using 3,3'-diaminobenzidine tetrahydrochloride as chromogen (Sigma, St. Louis, Mo.), and the sections were counterstained with hematoxylin.

Both positive (reactive lymph node) and negative (omitting the primary antibody) controls were run simultaneously.

ISEL of DNA fragments

Since extensive DNA fragmentation is an important characteristic of apoptosis, visualization of DNA breaks has proved useful in the identification of apoptotic cells [43]. This extensive DNA fragmentation results in a high density of 5'-protruding ends, which can be detected using the Klenow fragment of DNA polymerase I with a mixture of labeled nucleotides [48]. Briefly, the sections were deparaffinized and hydrated as described above. After incubation in 2×SSC buffer (80°C, 20 min) and protein digestion (500 µg/ml pronase in 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% SDS, at room temperature for 25 min), the sections were incubated with the Klenow fragment of *E. coli* DNA polymerase I under appropriate conditions (20 U/ml in 50 mM Tris HCl pH 7.5, 10 mM Mg_2Cl, 1 mM dithiothreitol, 250 µg/ml BSA with 100 µM of each dNTP, maintaining a proportion of 11-digoxigenin-dUTP/dTTP of 0.35/0.65; 2 h at 37°C). The dig-labeled DNA fragments were immunoenzymatically detected using an anti-digoxigenin polyclonal Fab fragment labeled with alkaline phosphatase (1/100 dilution, Boehringer-Mannheim, Germany); the enzymatic reaction was developed under microscopic control with nitroblue-tetrazolium and X-phosphate [12]. The sections were counterstained with diluted hematoxylin (25%), dehydrated, and mounted.

Both positive (reactive lymph node) and negative (omitting DNA polymerase in the enzymatic incubation) controls were simultaneously run.

Quantification of positive nuclei

At least 50 HPF (50 HPF=7.6 mm^2) were screened, or the complete lesion if smaller (18 patients, 12 without PCa and 6 with PCa), in each pathologic group. Both the number of positive nuclei per HPF and the number of neoplastic ductal cells intercepted by the microscope field diameter were recorded. The last score was used to estimate the number of neoplastic cells per HPF using the formula $N=(n\pi/4)^2$, where N is the number of estimated cells per HPF and n, the number of cells intercepted by the microscope field diameter [11, 25, 44]. The number of positive nuclei was always expressed per HPF and per 1,000 neoplastic cells. Both the average and the standard deviation (SD) values were calculated as representative scores per pathologic condition and patient.

The threshold of positivity was experimentally established at the positive control in each staining batch. Only those nuclei showing staining features similar to those of their corresponding positive control were considered positive for a given marker (MIB-1, ISEL). We only kept, for quantification, those nuclei in the same focus level taken as representative of any given HPF.

Slide cytometric analysis of DNA content

Feulgen-stained sections were used for DNA quantification [1], using the Cell Analysis System (CAS) model 200 and Quantitative DNA Analysis software (Becton-Dickinson). At least 200 nuclei were evaluated from each focus and the results recorded separately. From the same slide, both lymphocytes and nonneoplastic ductal cells were used as diploid controls. External diploid controls (rat hepatocytes provided by Becton-Dickinson) were included in each staining batch to normalize results. Several 5-µm sections were used for this analysis, according to previously published protocols that have proven valid in such material [19, 29, 46].

Only complete, nonoverlapping and focused nuclei were interactively selected, beginning in the most cellular area. The histogram of nuclear optical density was used to evaluate the DNA index (as referred to their corresponding diploid controls), the proliferation rate ($PR = S + G_2 + M / G_1 + S + G_2 + M$, expressed as a percentage), and the ratio between the nuclear area and the DNA content of the cells in each cell cycle phase. The last variable was also referred to the corresponding values in the nonneoplastic ductal cells of the prostate to normalize the results.

Statistical analysis

The average and SD of every variable in each pathologic condition (primary PIN, secondary PIN, and PCa) were statistically compared by means of Fisher's exact tests. The data were stratified in two subsets, above and below the corresponding threshold, in each pathologic group. The thresholds were 3% for MIB-1 immunoexpression, 0.4% for ISEL of DNA fragments, and 35 cells/HPF for cellularity estimation. Differences were considered statistically significant if $P<0.05$.

Results

Patients ages varied between 51 and 64 years (average 56) in the group without PCa and between 60 and

Table 1 Proliferative and apoptotic features in intraductal and invasive neoplasms of the prostate (*PIN* prostatic intraepithelial neoplasia, *PCa* prostatic carcinoma)

	Primary PIN (Mean ± SD)	Invasive PCa (Mean ± SD)	Secondary PIN (Mean ± SD)
M1B1 index	2.5±2.1	3.5±1.7	1.9±0.7
ISEL index	0.5±0.3	0.1±0.3	3.7±3.3
Cellularity	26.3±7.1	39.0±8.8	32.9±14.3

Table 2 Slide cytometric analysis of DNA content in intraductal and invasive neoplasms of the prostate

	Primary PIN (Mean ± SD)	Invasive PCa (Mean ± SD)	Secondary PIN (Mean ± SD)
Nuclear area (NuA) 33.04±5.66	42.80±6.34	41.11±4.25	
DNA index (DI)	1.11±0.16	1.12±0.11	1.22±0.07
NuA / DI	29.74±5.10	38.28±5.66	33.62±3.48

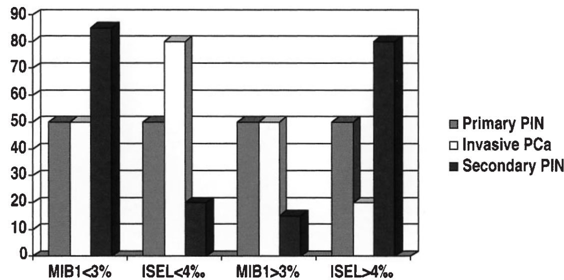

Fig. 1 Kinetic profiles in prostatic intraepithelial neoplasia (*PIN*) and low-grade prostatic carcinoma (*PCa*). Each *bar* represents the percentage of cases revealing a given feature (proliferation or apoptosis). The cases were categorized in two groups for both proliferation index (MIB-1, threshold 3%) and apoptotic index (in situ end-labeling [ISEL], threshold 4‰). The progression from primary PIN to low-grade PCa was kinetically defined by significant down-regulation of apoptosis with a moderate but not significant increase in proliferation. The opposite pattern characterized the transition primary PIN into secondary PIN. Both proliferation and apoptosis were significantly different in secondary PIN (Fisher's exact test)

71 years (average 68) in the group with PCa. All cases showed tufting or micropapillary high-grade PIN with no evidence of comedonecrosis, regardless of whether or not there was any association with PCa. The PCa was scored (Gleason) ≤4 in all patients. The MIB-1 index showed a progressive increase from secondary PIN through primary PIN to low-grade PCa, while the ISEL index revealed the lowest scores in low-grade PCa and the highest in secondary PIN (Table 1).

Combined analysis of kinetic indices in each pathologic condition showed a direct correlation between proliferation and apoptosis in primary PIN, while there was an inverse correlation between the two in both low-grade PCa and secondary PIN (Fig. 1). These opposite patterns were mainly due to very low apoptosis rates in low-grade PCa (in 80% of these cases ISEL indices were lower than 0.4%) and both relatively low PR and comparatively high ISEL in secondary PIN (Table 1, Figs. 1, 2). MIB-1 indices below 3% were observed in 86% of secondary PIN, and the ISEL index scored higher than 0.4% in 80% of them (Fig. 3). Those patterns resulted in the highest kinetic advantage for low-grade PCa; the kinetic index (PR-ISEL difference) in low-grade PCa (average, 3.4%) was 17-fold that in primary PIN (average, 0.2%) and was higher in both these conditions (low-grade PCa and primary PIN) than in secondary PIN (kinetic index, –1.8%).

The statistical analysis revealed no significant differences between primary PIN and low-grade PCa for either MIB-1 index (P=0.56) or ISEL index (P=0.09). Although a discrete increase in the MIB-1 index was observed in low-grade PCa (Table 1), the cases were equally distributed around (50% below and 50% above) 3% (Fig. 1). Both primary PIN and low-grade PCa showed low ISEL indices, which were lower in low-grade PCa than in primary PIN but with wide intergroup overlapping, precluding any statistical conclusion (Table 1).

Anyway, the progression primary PIN → low-grade PCa was kinetically related to down-regulation of apoptosis: 80% of cases of low-grade PCa showed an ISEL index ≤0.4%, while only 50% of primary PIN revealed similar scores (Fig. 1). That tendency can only be confirmed in groups with less variability and with more cases included in the analysis.

On the other hand, secondary PIN showed statistically significant kinetic features from both primary PIN and low-grade PCa (P<0.0001 in all comparisons, for both MIB-1 and ISEL indices). Secondary PIN was kinetically characterized by the lowest MIB-1 index and the highest ISEL index (Table 1), the reverse pattern of low-grade PCa (Fig. 1), which was characterized by low proliferation (MIB-1 index ≤3% in 86% of cases) and high apoptosis (ISEL index >0.4% in 80% of cases).

The slide cytometric analysis of DNA content revealed at least two G_0/G_1 cells in all cases but 4 (2 primary PIN and 2 invasive low-grade PCa). The average DNA content of the prevalent G_0/G_1 peak showed a progressive increase from primary PIN through invasive low-grade PCa to secondary PIN (Table 2), with diploid G_0/G_1 cells predominating in primary PIN and low-grade PCa and hyperdiploid G_0/G_1 cells in secondary PIN (Fig. 4). Both the nuclear area and the nuclear area / DNA index ratio revealed the highest score for invasive low-grade PCa and the lowest for primary PIN (Table 2). The presence of multiple cases with two G_0/G_1 peaks precluded an appropriate and reliable evaluation of proliferative index from the DNA histograms in each group.

Low-grade PCa displayed the highest cellularity scores, whereas primary PIN had the lowest scores and secondary PIN revealed average values between those for primary PIN and low-grade PCa, but with the highest variability (Table 1). No differences were detected in a comparison between low-grade PCa and secondary PIN. Low-grade PCa showed slightly higher cellularity than secondary PIN, but the high variability of the latter resulted in a

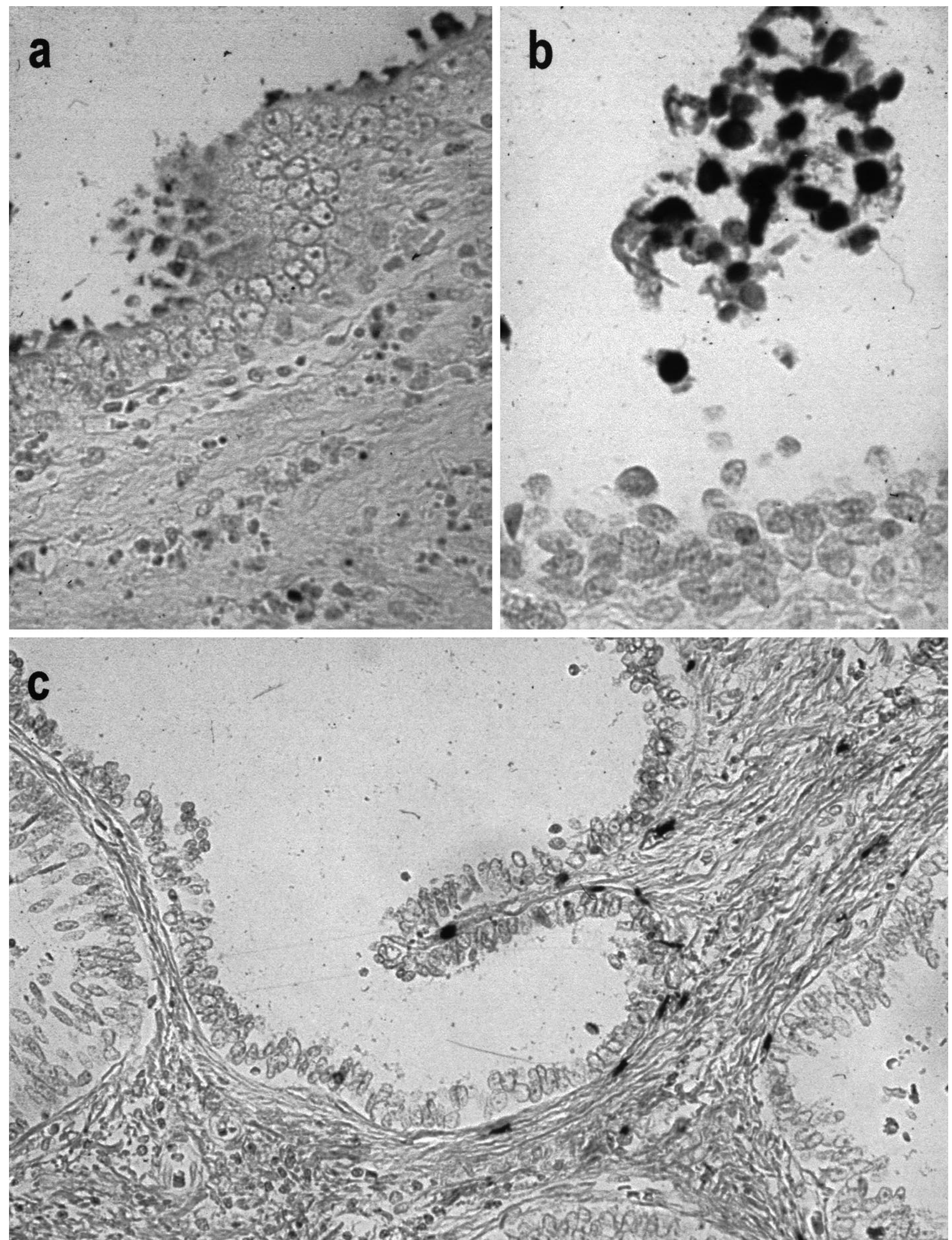

Fig. 2 In situ end-labeling in **a** primary PIN, **b** secondary PIN, and **c** low-grade PCa. Very low signal was detected in ductal cells affected by either primary PIN or low-grade PCa, whereas significantly higher values were obtained in secondary PIN. Note the positive signal provided by stromal and endothelial cells in **c** (internal control). **a**, **b** ×400; **c** ×200

broad undefined window precluding any statistical conclusion. Even after cellularity categorization, no differences were detected: 50% of low-grade PCa and 60% of secondary PIN revealed scores of ≤35 cells/HPF (P=0.1004). However, primary PIN was proven statistically different from both low-grade PCa and secondary PIN (P<0.0001 in both cases). Primary PIN showed the lowest cellularity of all groups (Table 1), 80% of them had ≤35 cells/HPF.

Discussion

Two main inferences could be drawn in this study. First, PIN is kinetically different according as whether there is coexistent PCa. Secondly, apoptosis plays an essential role in early steps of prostate tumorigenesis, while proliferation seems to be of secondary importance in this progression. We discuss these points below.

Different cell kinetic profiles characterized PIN associated and not associated with coexistent PCa. The relatively low proliferation and relatively high apoptosis of PIN with coexistent PCa (secondary PIN) were demonstrably statistically different from those of PIN with no PCa (primary PIN). This differential kinetic evolution suggests that they represent biologically different conditions, although with similar morphology. High-grade PIN is a neoplasm of uncertain biologic behavior, which occurs as a primary disease but exists most often in asso-

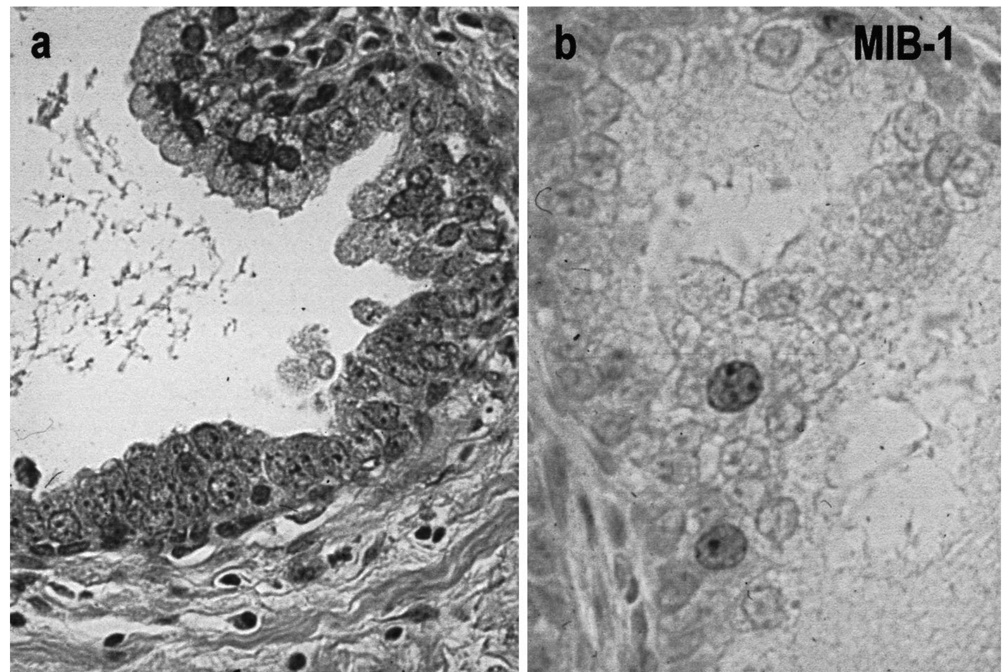

Fig. 3a, b Primary PIN. Relatively low proliferation rate characterized PIN, as detected by nuclear staining with MIB-1 antibody. **a** H&E, ×250; **b** MIB-1, ×400

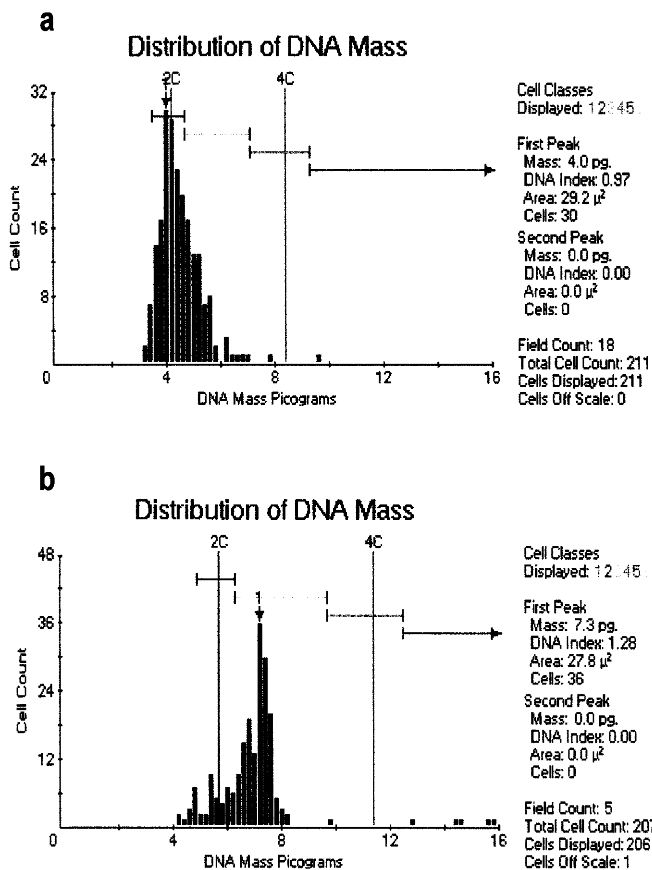

Fig. 4 DNA histogram patterns in **a** primary and **b** secondary PIN. Diploid G_0/G_1 cells were frequently found in primary PIN and low-grade PCa, while secondary PIN revealed hyperdiploid G_0/G_1 cells (peak labeled *1* in both *panels*)

ciation with invasive PCa; its clinicopathological correlates remain to be defined. The histological and biological information on high-grade PIN is based mainly on case series of secondary PIN, and there are no papers addressing the kinetic and biologic differences between primary and secondary PIN. Our results display two distinctive kinetic profiles for them. Primary PIN cases were evenly distributed around MIB-1 and ISEL thresholds (50% above and 50% below), whereas a decreasing PR (86% of cases had an MIB-1 index <3%) and increasing ISEL index (80% of cases showed an ISEL index >0.4%) characterized secondary PIN (Figs. 2, 4). The kinetic pattern of low-grade PCa was the opposite of that observed in secondary PIN (relatively high proliferation and comparatively low apoptosis) and closer to that of primary PIN than to that of secondary PIN. When all these features are considered together, the most likely kinetic evolution of secondary PIN (Fig. 4) seems to be from primary PIN (divergent progression, invasive and intraductal) rather than from low-grade PCa (linear progression, primary PIN → low-grade PCa → secondary PIN).

The kinetic features of secondary PIN are consistent with an evolved tumor cell stage with regressive features (low proliferation and high apoptosis). Actually, in situ genetic analysis of coexistent PIN and PCa foci often shows similar chromosomal anomalies, although several PIN foci have been demonstrated to have more alterations than matched carcinoma foci [39]. Preliminary results from a comparative genomic hybridization analysis of PIN from this series revealed more extensive genetic abnormalities in secondary PIN and the coexistent PCa (20q12, 12q14, 9q22, and 16p12) than in primary PIN (20q and 16p) [28]. Those findings suggest that PIN foci can undergo more extensive chromosome evolution than

PCa and would also support a potential multifocality in the evolution of PCa [2, 3, 6]. Likewise, they point to an independent clonal evolution of secondary PIN cells, different from that of the sequence primary PIN → low-grade PCa [28]. A progressive accumulation of genetic abnormalities in PIN would increase the probability of genetically lethal events in those tumor cells, thus rising the apoptosis rate. The increase in the cell loss determines a reduction in the pool of mitotically active cells and, therefore, lowers PR. However, this up-regulated apoptosis pattern is not unique to high-grade PIN and has also been described in ductal carcinoma in situ (DCIS) of the breast. Harn et al. [21] reported significantly higher ISEL indices in breast DCIS than in either invasive ductal carcinomas or metastatic breast carcinomas, strongly correlated with abnormal p53 expression in the intraductal component. Multiple deletions involving several tumor suppressor genes are relatively frequent findings in intraepithelial neoplasms [17, 20, 21, 41]. We therefore propose that a distinction be made between primary and secondary PIN, in a similar way to the distinction in terminology that is widely accepted in bladder pathology [37, 49, 50]. This would mean interpreting secondary PIN as part of the evolution of prostate carcinoma rather than as a precursor, as suggested in some clinicopathological studies [30, 42].

Our results also emphasize the key role of apoptosis in early prostatic neoplasms. Initially, the association of high-grade PIN with low-grade PCa might be surprising, but previous reports have demonstrated it [38, 40] and we must consider that PIN grading is based on cytological features while Gleason grading relies only on architectural parameters and most PCa show a high nuclear grade. Therefore, that transition is not so surprising, although the volume of PIN is positively correlated with Gleason score [40]. The apoptosis analysis, contrasted by DNA-ploidy and ISEL of fragmented DNA, showed extremely low indices in low-grade PCa (80% of low-grade PCa displayed ISEL index <0.4% and revealed the highest nuclear area/DNA index ratio), smaller than in primary PIN. At the kinetic level, these two conditions were proven to be statistically different from secondary PIN, essentially as the result of apoptosis down-regulation. Under physiologic control, apoptosis closely correlates with proliferation to maintain a constant cell number [27]. A continuous increase in proliferation markers normally defines tumor progression, with a parallel increase in apoptosis [43]. Prior to any significant difference in proliferation markers, intraepithelial lesions seem to down-regulate apoptosis, resulting in a kinetic advantage and monoclonal expansions specifically of these lesions, as has been shown in breast DCIS [12], benign adrenal cortical proliferative lesions [8, 13], and adrenal medullary hyperplasia [10, 16] or C-cell hyperplasias in MEN-2A [9, 14, 51]. Decreased apoptotic cell loss would increase the cellular pool and allow the accumulation of genetically damaged cells, ending in a convergent cellular selection. That selection process would explain both tumor progression and heterogeneity, whether or not related with androgen levels or with androgen receptor abnormalities [26, 32, 34, 35].

Previous reports showed a progressive and variable increase of apoptosis in PIN and PCa, but with SDs equal to or higher than the average [18], precluding any statistical assessment. This finding has been proposed as an indication of the continuum, or pathway, leading to PCa. However, apoptotic indices are normally low and require screening of several HPF to achieve reliable results. This is especially true for heterogeneous conditions, such as PCa. That inherent heterogeneity determines group overlapping and makes it difficult (and sometimes impossible) to reach statistical conclusions.

Proliferation parameters have been shown to be useful in the diagnosis of intraepithelial lesions (distinction of atypical hyperplasias from carcinomas in situ) and directly correlated with tumor grading [5, 7, 23, 36]. Our proliferation results suggest a kinetic difference between primary and secondary PIN: only secondary PIN was proven to be statistically different from both primary PIN and low-grade PCa, with higher scores in the latter conditions than in secondary PIN (Table 1). We have found a similar kinetic pattern in CIS associated with muscle invasive transitional cell carcinoma of the urinary bladder: significantly decreased proliferative indices were revealed in the intraepithelial compartment than in the invasive one [17].

Our results are in the range of grade-1 PCa reported by Helpap [23], and both his and ours are lower than those reported by Tamboli et al. [45]. These last authors did not provide MIB-1 indices by tumor grade and stage, but all their tumors had Gleason's scores of 5+, and over one-third of them were stage III–IV. That is a different series distribution from ours, which showed a combined Gleason score of ≤4. The MIB-1 indices previously reported for PIN are in the range of our scores in primary PIN but reveal high variability (the SD was 1.5-fold the corresponding average) [45], probably an expression of tumor heterogeneity. Both intratumoral heterogeneity and the quantitation method will contribute to these discrepant results. We screened 50 HPF/focus (or the complete lesion if smaller) and 5 different foci per patient and pathologic condition to decrease the score variability (the smaller the SD the higher the measurement accuracy) and to take the intratumoral heterogeneity into consideration. Our SD values were always below average, suggesting the use of a better sampling method for tumor evaluation than for previously reported results [23, 45]. Studies in other organs have demonstrated a topographic heterogeneity of kinetic features missed with the standard screening methods [11, 16].

In summary, two kinetic profiles could be drawn in PIN, depending on whether or not it was associated with coexistent low-grade PCa, which suggests that these represent biologically different conditions. The unique kinetic profile of secondary PIN, with relatively low proliferation and relatively high apoptosis, is consistent with an evolved tumor cell stage with regressive features rather than with a precursor lesion. Early steps in tumorigen-

esis in low-grade prostate carcinomas are kinetically characterized by down-regulated apoptosis rather than by increased proliferation.

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